HOOKWORM VACCINE

This application claims priority to International patent application PCT/US02/33106 (filed 17 October, 2002, of which it is a continuation-in-part), and to US provisional patent applications 60/329,553 (filed 17 October, 2001), 60/332, 007 (filed 23 November 2001) 60,375,404 (filed 26 April 2001), and 60/505,848 (filed 26 September 2003). The entire contents of each application to which priority is claimed is hereby incorporated by reference.

This invention was made in part by funds from government grants: Tropical Medicine Research Center (TMRC) grant from the National Institutes of Health P50 A1-39461 and A1-32726. The United States government may have rights in this invention.

DESCRIPTION

BACKGROUND OF THE INVENTION

Field of the Invention

The invention generally relates to a vaccine for hookworm. In particular, the invention provides vaccines based on parasite-derived antigens.

Background of the Invention

Hookworm infection is a significant public health concern in developing countries around the world, causing enteritis, intestinal blood loss, anemia, developmental delays, and malnutrition. It is estimated that there are more than one billion cases of human hookworm infection worldwide, with 194 million cases in China alone (Hotez et al. 1997). In some regions of China such as Hainan Province in the South China Sea more than 60 percent of the population harbors hookworms (Gandhi et al. 2001).

Most of the pathology caused by hookworm results from the adult stages of the parasite in the human intestine. The attachment of adult *Ancylostoma* and *Necator* hookworms to the mucosa and submucosa of the vertebrate small intestine is one of the best-defined examples of host-parasite relationships in all of parasitology. Comprised of several cubic millimeters of host mucosal and submucosal tissue lodged in the buccal capsule of the parasite, it is possible to

actually touch the host-parasite relationship at necropsy or autopsy (Kalkofen, 1970; Kalkofen, 1974).

The dog hookworm *Ancylostoma caninum* is a major cause of morbidity and mortality in dogs throughout the world including subtropical regions of North America. Hookworm-associated blood loss leading to severe anemia and even death can occur in dogs between 2 and 3 weeks after a single primary infection (Soulsby, 1982; Jones and Hotez, 2002). Significantly, *A. caninum* has also been recently identified as an important human pathogen. Zoonotic infection with one adult *A. caninum* parasite can result in eosinophilic enteritis syndrome, an inflammatory condition of the intestine in response to invasion by the parasite (Prociv and Croese, 1990). The pathogenesis of *A. caninum* infection is associated with the intestinal blood loss that occurs during adult worm attachment and feeding in the mammalian small intestine (Kalkofen, 1970; Kalkofen, 1974).

Current efforts for the treatment and control of hookworm infestations are limited to periodic removal of adult hookworms from patients with anthelmintics. This approach has several limitations, including rapid reinfection following treatment, requiring multiple visits, and the eventual development of anthelmintic resistant strains of hookworms following several years of heavy anthelmintic treatments (Savioli et al. 1997; Geerts and Gryseels, 2000). Thus, it would be of great benefit to have available additional methods for both treating and preventing hookworm infection in mammals. For example, it would be highly advantageous to have available vaccines to treat or prevent hookworm infection.

SUMMARY OF THE INVENTION

The present invention provides preparations for eliciting an immune response against hookworm. The preparations contain various hookworm antigens which have been identified as useful for eliciting an immune response. These preparations may be used as vaccines against hookworm in mammals, for example, in humans. As a result of the administration of the preparations, the vaccinated mammal may develop an immune response against hookworm which causes immunity to infection by the parasite, or may display a lower worm burden, decreased blood loss, or a decrease in size of parasitizing hookworms. To that end, the invention provides a composition comprising a recombinant or synthetic antigen or a fragment

thereof derived from hookworm, and a pharmacologically acceptable carrier. The recombinant or synthetic antigen may display at least about 80% identity to an antigen such as ASP-1, ACE, CTL, APR-1, APR-2, TMP, MEP-1, MEP-2, ASP-1, ASP-2, ASP-3, ASP-4, ASP-5, ASP-6, TTR-1, TTR-2, 103 (also referred to as SAA-1), 16, VWF, CTL, API, MTP-1, MTP-2, MTP-3, FAR-1, KPI-1, APR-1, APR-2, AP, ASP-1, ASP-2, API, CP-1, CP-2, CP-3, CP-4, CYS, and GST. In preferred embodiments, the antigen is ASP-1, ASP-2, MTP-1, 103 (SAA), 16, GST, TMP, MEP-1, APR or CP-2. The antigens may be derived from a hookworm from species such as *Necator americanus*, *Ancylostoma caninum*, *Ancylostoma ceylanicum*, and *Ancylostoma duodenale*.

The invention also provides a method of eliciting an immune response to hookworm in a mammal. The method includes the step of administering to the mammal an effective amount of a composition comprising a recombinant or synthetic antigen (or a fragment of the antigen) derived from hookworm, and a pharmacologically acceptable carrier. The recombinant or synthetic antigen may display at least about 80% identity to an antigen such as ASP-1, ACE, CTL, APR-1, APR-2, TMP, MEP-1, MEP-2, ASP-1, ASP-2, ASP-3, ASP-4, ASP-5, ASP-6, TTR-1, TTR-2, 103 (also referred to as SAA-1), 16, VWF, CTL, API, MTP-1, MTP-2, MTP-3, FAR-1, KPI-1, APR-1, APR-2, AP, ASP-1, ASP-2, API, CP-1, CP-2, CP-3, CP-4, CYS, and GST. In preferred embodiments, the antigen is ASP-1, ASP-2, MTP-1, 103 (SAA), 16, GST, TMP, MEP-1, APR, or CP-2. The antigens may be derived from a hookworm from species such as *Necator americanus*, *Ancylostoma caninum*, *Ancylostoma ceylanicum*, and *Ancylostoma duodenale*.

The invention further provides a method of vaccinating a mammal against hookworm. The method includes the step of administering to the mammal an effective amount of a composition comprising a recombinant or synthetic antigen (or a fragment of the antigen) derived from hookworm and a pharmacologically acceptable carrier. The recombinant or synthetic antigen may display at least about 80% identity with an antigen such as ASP-1, ACE, CTL, APR-1, APR-2, TMP, MEP-1, MEP-2, MTP-1, ASP-1, ASP-2, ASP-3, ASP-4, ASP-5, ASP-6, TTR-1, TTR-2, 103 (also referred to as SAA-1), 16, VWF, CTL, API, MTP-1, MTP-2, MTP-3, FAR-1, KPI-1, APR-1, APR-2, AP, ASP-1, ASP-2, MTP-1, API, CP-1, CP-2, CP-3, CP-4, CYS, and GST. In preferred embodiments, the antigen is ASP-1, ASP-2, MTP-1, 103 (SAA), 16, GST, TMP, MEP-1, APR, or CP-2. The antigens may be derived from a hookworm

from species such as *Necator americanus*, *Ancylostoma caninum*, *Ancylostoma ceylanicum*, and *Ancylostoma duodenale*.

The invention further provides a composition comprising a recombinant or synthetic antigen (or a fragment of the antigen) derived from hookworm. The recombinant or synthetic antigen display at least about 80% identity with an antigen such as ASP-1, ACE, CTL, APR-1, APR-2, TMP, MEP-1, MEP-2, ASP-1, ASP-2, ASP-3, ASP-4, ASP-5, ASP-6, TTR-1, TTR-2, 103 (also referred to as SAA-1), 16, VWF, CTL, API, MTP-1, MTP-2, MTP-3, FAR-1, KPI-1, APR-1, APR-2, AP, ASP-1, ASP-2, API, CP-1, CP-2, CP-3, CP-4, CYS, and GST. The composition further comprises a pharmacologically acceptable carrier. In preferred embodiments, the antigen is ASP-1, ASP-2, MTP-1, 103 (SAA), 16, GST, TMP, MEP-1, APR, or CP-2. The antigens may be derived from a hookworm from species such as *Necator americanus*, *Ancylostoma caninum*, *Ancylostoma ceylanicum*, and *Ancylostoma duodenale*.

The invention further provides a vaccine comprising a recombinant or synthetic antigen (or a fragment of the antigen) derived from hookworm. The recombinant or synthetic antigen displays at least about 80% identity with an antigen such as ASP-1, ACE, CTL, APR-1, APR-2, TMP, MEP-1, MEP-2, ASP-1, ASP-2, ASP-3, ASP-4, ASP-5, ASP-6, TTR-1, TTR-2, 103 (also referred to as SAA-1), 16, VWF, CTL, API, MTP-1, MTP-2, MTP-3, FAR-1, KPI-1, APR-1, APR-2, AP, ASP-1, ASP-2, API, CP-1, CP-2, CP-3, CP-4, CYS, and GST. The vaccine further comprises a pharmacologically acceptable carrier. In preferred embodiments, the antigen is ASP-1, ASP-2, MTP-1, 103 (SAA), 16, GST, TMP, MEP-1, APR, or CP-2. The antigens may be derived from a hookworm from species such as *Necator americanus*, *Ancylostoma caninum*, *Ancylostoma ceylanicum*, and *Ancylostoma duodenale*.

The present invention further provides a composition for eliciting an immune response comprising a recombinant or synthetic antigen (or a fragment of the antigen) derived from hookworm. The recombinant or synthetic antigen displays at least about 80% identity with an antigen selected from the group consisting of ASP-1, ACE, CTL, APR-1, APR-2, TMP, MEP-1, MEP-2, ASP-1, ASP-2, ASP-3, ASP-4, ASP-5, ASP-6, TTR-1, TTR-2, 103 (also referred to as SAA-1), 16, VWF, CTL, API, MTP-1, MTP-2, MTP-3, FAR-1, KPI-1, APR-1, APR-2, AP, ASP-1, ASP-2, API, CP-1, CP-2, CP-3, CP-4, CYS, and GST. The composition further comprises a pharmacologically acceptable carrier. In preferred embodiments, the antigen is ASP-1, ASP-2, MTP-1, 103 (SAA), 16, GST, TMP, MEP-1, APR, or CP-2. The antigens may

be derived from a hookworm from species such as *Necator americanus*, *Ancylostoma caninum*, *Ancylostoma ceylanicum*, and *Ancylostoma duodenale.*

The invention further provides a method for enabling vaccination of a patient against parasite derived infectious diseases. The method includes the steps of treating hookworm infection to a degree sufficient to increase lymphocyte proliferation, and vaccinating the patient against an infectious disease such as HIV, tuberculosis, malaria, measles, tetanus, diphtheria, pertussis, or polio.

The present invention also provides a method for enabling hookworm vaccination. The method includes the steps of chemically treating a hookworm infected patient to ameliorate hookworm infection, and vaccinating the patient with a recombinant or synthetic antigen (or a fragment of the antigen) derived from hookworm after amelioration of hookworm infection. In the method, the hookworm infection may be completely eradicated by treatment, or may be lessened to such an extent that hookworm vaccination is effective. The recombinant or synthetic antigen may display at least about 80% identity with an antigen such as ASP-1, ACE, CTL, APR-1, APR-2, TMP, MEP-1, MEP-2, ASP-1, ASP-2, ASP-3, ASP-4, ASP-5, ASP-6, TTR-1, TTR-2, 103 (also referred to as SAA-1), 16, VWF, CTL, API, MTP-1, MTP-2, MTP-3, FAR-1, KPI-1, APR-1, APR-2, AP, ASP-1, ASP-2, API, CP-1, CP-2, CP-3, CP-4, CYS, and GSTThe antigens may be derived from a hookworm from species such as *Necator americanus*, *Ancylostoma caninum*, *Ancylostoma ceylanicum*, and *Ancylostoma duodenale*..

The present invention also provides a method for reducing blood loss in a patient infected with hookworm. The method includes the step of administering to the patient a composition comprising a recombinant or synthetic antigen (or a fragment of the antigen) derived from hookworm, and a pharmacologically acceptable carrier.

The present invention also provides a method for reducing hookworm size in a patient infected with hookworm. The method includes the step of administering to the patient a composition comprising a recombinant or synthetic antigen (or a fragment of the antigen) derived from hookworm, and a pharmacologically acceptable carrier.

The invention further provides a method of reducing hookworm burden in a patient infected with hookworm. The method comprises the step of administering to the patient a composition comprising a recombinant or synthetic antigen (or a fragment of the antigen) derived from hookworm, and a pharmacologically acceptable carrier.

The present invention also provides the following nucleic acid and amino acid sequences: SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63 and SEQ ID NO: 64.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A and B. Na-ASP-1: A, cDNA (SEQ ID NO: 1) and B, deduced amino acid sequence (SEQ ID NO: 2). GeneBank accession # AF079521.

Figure 2A and B. Na-ACE: A, cDNA (SEQ ID NO: 3) and B, deduced amino acid sequence (SEQ ID NO: 4). GeneBank accession # AF536813.

Figure 3A and B. Na-CTL: A, cDNA (SEQ ID NO: 5) and B, deduced amino acid sequence (SEQ ID NO: 6).

Figure 4A and B. Na-APR-1: A, cDNA (SEQ ID NO: 7) and B, deduced amino acid sequence (SEQ ID NO: 8).

Figure 5A and B. Na-APR-2: A, cDNA (SEQ ID NO: 9) and B, deduced amino acid sequence (SEQ ID NO: 10).

Figure 6A and B. Ac-TMP: A, cDNA (SEQ ID NO: 11) and B, deduced amino acid sequence (SEQ ID NO:12).

Figure 7A and B. Ac-MEP-1: A, cDNA (SEQ ID NO: 13) and B, deduced amino acid sequence (SEQ ID NO:14). GeneBank accession # AF273084.

Figure 8A and B. Ac-MTP-1: A, cDNA (SEQ ID NO: 15) and B, deduced amino acid sequence (SEQ ID NO: 16). GeneBank accession # AY036056.

Figure 9A and B. Ac-ASP-1: A, cDNA (SEQ ID NO: 17) and B, deduced amino acid sequence (SEQ ID NO: 18). GeneBank accession # AF132291.

Figure 10A and B. Ac-ASP-2: A, cDNA (SEQ ID NO: 19) and B, deduced amino acid sequence (SEQ ID NO: 20). GeneBank accession # AF089728.

Figure 11A and B. Ac-ASP-3: A, cDNA (SEQ ID NO: 21) and B, deduced amino acid sequence (SEQ ID NO: 22).

Figure 12A and B. Ac-ASP-4: A, cDNA (SEQ ID NO: 23) and B, deduced amino acid sequence (SEQ ID NO: 24).

Figure 13A and B. Ac-ASP-5: A, cDNA (SEQ ID NO: 25) and B, deduced amino acid sequence (SEQ ID NO: 26).

Figure 14A and B. Ac-ASP-6: A, cDNA (SEQ ID NO: 27) and B, deduced amino acid sequence (SEQ ID NO: 28).

Figure 15 A and B. Ac-TTR-1: A, cDNA (SEQ ID NO: 29) and B, amino acid sequence (SEQ ID NO:30) deduced from nucleotides 25-531.

Figure 16A and B. Ac-103: A, cDNA (SEQ ID NO: 31) and B, amino acid sequence (SEQ ID NO: 32).

Figure 17A and B. Ac-VWF: A, cDNA (SEQ ID NO: 33) and B, amino acid sequence (SEQ ID NO: 34).

Figure 18A and B. Ac-CTL: A, cDNA (SEQ ID NO: 35) and B, amino acid sequence (SEQ ID NO: 36).

Figure 19A and B. Ac-API-1: A, cDNA (SEQ ID NO: 37) and B, amino acid sequence (SEQ ID NO: 38) deduced from nucleotides 23-706.

Figure 20A and B. Ac-MTP-1: A, cDNA (SEQ ID NO: 39) and B, amino acid sequence (SEQ ID NO: 40).

Figure 21A and B. Ac-MTP-2: A, cDNA (SEQ ID NO: 41) and B, amino acid sequence (SEQ ID NO: 42).

Figure 22A and B. Ac-MTP-3: A, cDNA (SEQ ID NO: 43) and B, amino acid sequence (SEQ ID NO: 44).

Figure 23A and B. Ac-FAR-1: A, cDNA (SEQ ID NO: 45) and B, amino acid sequence (SEQ ID NO: 46). GeneBank Acession # AF529181

Figure 24A - C. Ac-KPI-1: A and B, cDNA (SEQ ID NO: 47) and C, amino acid sequence (SEQ ID NO: 48) deduced from nucleotides 12-2291.

Figure 25A and B. Ac-APR-1: A, cDNA (SEQ ID NO: 49) and B, amino acid sequence (SEQ ID NO: 50).

Figure 26A and B. Ac-APR-2: A, partial cDNA sequence (SEQ ID NO: 51) and B, partial amino acid sequence (SEQ ID NO: 52).

Figure 27A and B. Ac-AP: A, cDNA (SEQ ID NO: 53) and B, amino acid sequence (SEQ ID NO: 54).

Figure 28A and B. Ay-ASP-1: A, cDNA (SEQ ID NO: 55) and B, amino acid sequence (SEQ ID NO: 56).

Figure 29A and B. Ay-ASP-2: A, cDNA (SEQ ID NO: 57) and B, amino acid sequence (SEQ ID NO: 58).

Figure 30A and B. Ay-MTP-1: A, cDNA (SEQ ID NO: 59) and B, amino acid sequence (SEQ ID NO: 60).

Figure 31A and B. Ay-API-1: A, cDNA (SEQ ID NO: 61) and B, amino acid sequence (SEQ ID NO: 62) deduced from nucleotides 23-703.

Figure 32A and B. Ay-TTR: A, partial cDNA (SEQ ID NO: 63) and B, partial amino acid sequence (SEQ ID NO: 64).

Figure 33A and B. Spearman rank order correlations between hookworm burden and anti-MTP-1 antibody titer. A) total worms; B) median EPG.

Figure 34A-C. Antigen-specific geometric mean IgG1 antibody titers in dogs vaccinated with *A. caninum* recombinant fusion proteins as a function of time. Geometric means were calculated for a total of 6 dogs in each group, except for Ac-AP in which only a single dog developed an antigen-specific antibody response. The arrows denote timed vaccinations. (A) Anti-Ac-APR-1 responses (n=6). (B) Anti-Ac-TMP responses (n=6). (C) Anti-Ac-AP responses (n=1).

Figure 35. Female and male adult *A. caninum* hookworms recovered from the colons of either vaccinated or alum-injected dogs.

Figure 36A and B. Spearman rank order correlations between hookworm burden and anti-MTP-1 antibody titer

Figure 37A and B. A) Relationship between anti-TTR IgE antibodies and hookworm burden reductions; B) Relationship between anti-TTR IgG1 antibodies and hookworm burden reductions

Figure 38A and B. HV-4 Canine hemoglobin (B) and hematocrit (A) changes following L3 challenge

Figure 39. Statistically significant reduction in worm size (between 1 and 2 mm) among the TTR vaccinated group relative to the adjuvant control group.

Figure 40. CD4+ lymphocytes from hookworm-infected (egg positive) individual post-stimulation with Ancylostoma L3 antigen.

Figure 41. CD4+ lymphocytes from hookworm-infected (egg positive) individual post-stimulation with Pichia-expresses recombinant Na-ASP-1.

Figure 42. Alignment of deduced amino acid sequences of *Ancylostom*a-secreted protein (ASP)–1 derived from different species of third-stage hookworm larvae. Sequences were aligned by use of CLUSTAL W software and were prepared for display by use of BOXSHADE software. Black boxes, identical amino acids; *gray boxes,* similar amino acids; *asterisks,* amino acids common to every sequence; and *arrows,* cysteines conserved in all ASPs. Names and GenBank accession nos. are as follows: *Ay (A. ceylanicum)*–ASP-1 (SEQ ID NO: 56), AAN11402; *Ac (A. caninum)*–ASP-1 (SEQ ID NO: 18), AAC47001; *Ad (A. duodenale)*–ASP-1, AAD13339 (SEQ ID NO: 67); and *Na (Necator americanus)*–ASP-1 (SEQ ID NO: 2), AAD13340. The amino acid sequence identities between *Ay*-ASP-1 and other hookworm ASP-1 proteins are shown at the end of sequence.

Figure 43A and B. A, Alignment of deduced amino acid sequences of *Ancylostom*a-secreted protein (ASP)–2 derived from different species of third-stage hookworm larvae. Sequences were aligned by use of CLUSTAL W software and were prepared for display by use of BOXSHADE software. *Black boxes,* dentical amino acids; *gray boxes,* similar amino acids; *asterisks,* amino acids common to every sequence; and *arrows,* cysteines conserved in all ASPs. The names and GenBank accession nos. are as follows: *Ay (A. ceylanicum)*–ASP-2 (SEQ ID NO: 58), AAP41953; *Ac (A. caninum)*–ASP-2 (SEQ ID NO: 20), AAC35986; Ad (A. duodenale)–ASP-2 (SEQ ID NO: 68), AAP41951; and *Na (Necator americanus)*–ASP-2 (SEQ ID NO: 69), AAP41952. The amino acid sequence identities between *Ay*-ASP-2 (SEQ ID NO:

58) and other hookworm ASP-2 proteins are shown at the end of sequence. B, cDNA sequence of Na-ASP-2 (SEQ ID NO: 82).

Figure 44. Total IgG titers (geometric) in serum from golden Syrian hamsters vaccinated with *Ay (Ancylostoma ceylanicum)*–ASP-1 (SEQ ID NO: 56) mean SD *(Ancylostoma*-secreted protein) and *Ay*-ASP-2 (SEQ ID NO: 58) formulated with either Quil A or Montanide ISA-720 as adjuvant. Serum samples were obtained 8 days after the final vaccination (see Materials and Methods). Vaccinations with radiation-attenuated *A. ceylanicum* third-stage infective larvae (irL3) are included as a positive control (hamsters/group). Antibody titers were determined by measuring the last dilution that resulted in 3 SD above *n* p 10 background.

Figure 45. The relationship between age and prevalence (bars) and log transformed eggs per gram of feces (lnepg) (●) in people infected with *Necator americanus* in Minas Gerais, Brazil *(n* = 495) and Hainan Province, China *(n* = 396). Lines represent standard error of the mean for lnepg.

Figure 46. Secretion, purification and biochemical analysis of recombinant *Ac*-ASP-2 (SEQ ID NO: 20) expressed in S*f*9 insect cells. The purified protein displayed a mass of 24,492 da (major species) by mass spectroscopy with smaller quantities of minor species observed between 24,592 and 25,537 da

Figure 47. The distribution of anti-ASP-2 serum antibody isotypes from people in hookworm-endemic areas of Hainan Province, China *(n* = 222) and Minas Gerais, Brazil *(n* = 285) Antibody isotypes not shown here were not detected against ASP-2. Restriction in the antibody subclass response to ASP-2.

Figure 48. The relationship between antibody isotype responses to ASP-2 and intensity of infections with *Necator americanus*. The relationship between individuals with IgE (IgE-pos) or without IgE (IgE-neg) against ASP-2 and fecal egg counts in samples from Hainan Province, China (a) and Minas Gerais, Brazil (b). Bars indicate 95% confidence intervals for the mean fecal egg counts. *P* values and percentages indicate differences in mean fecal egg counts between IgE-positive and IgE-negative groups.

Figure 49. Canine anti-ASP-2 antibodies induced by vaccination recognize recombinant and parasite-derived ASP-2. Geometric mean titers of the IgG1 (□), IgG2 (●) and IgE (▲) antibody responses against ASP-2 in canines vaccinated with recombinant ASP-2 The control group was vaccinated with AS03 adjuvant only and had no titers (data not shown). The letter C inside a

gray arrow refers to larval challenge; the letter N inside a gray arrow refers to necropsy. Individual dogs (A-E) vaccinated with recombinant ASP-2 generated antibodies at day 75 (before larval challenge) that immunoprecipitated native ASP-2 from L3 extracts.

Figure 50. Vaccination of dogs with recombinant ASP-2 provides protection against hookworm infection. Fecal egg counts for canines vaccinated with ASP-2 or the adjuvant AS03 alone (con) (a). Comparison of adult worms retrieved during necropsy from the colon and small intestine of canines vaccinated with ASP-2/AS03 and AS03 alone (con) (b). Bars indicate standard error of the mean for each group. Sera from dogs immunized with ASP-2 partially inhibited migration of *A. caninum* third stage larvae through canine skin *in vitro* (c). There was a 30% reduction ($P = 0.02$) in the numbers of L3 that penetrated canine skin when L3 were first incubated in sera from vaccinated dogs compared to control animals. Values for inhibition assays are raw data.

Figure 51. pH profile of the catalytic activity of recombinant *Ac*-CP-2 against the substrate Z-Phe-Arg-AMC.

Figure 52. The geometric mean titers of the IgG1 (A) and IgG2 (B) antibody responses of vaccinated dogs against recombinant *Ac*-CP-2 formulated with ASO3 (■), ASO2 (●), ISA70 (□), alum (▲) or alum alone without CP-2 (○). Open arrows on the X-axis indicate the days of vaccination (numbers inside) and larval challenge (C).

Figure 53. The geometric mean egg counts from dogs immunized with *Ac*-CP-2 formulated with different adjuvants or alum alone (control). The error bars refer to standard error of the mean. The numbers within the bars refer to the *P*-value of a Dunnett (Post Hoc) test, a pairwise multiple comparison *t* test that compares a set of treatments against a single control mean.

Figure 54. The proportions of male to female worms recovered from dogs immunized with *Ac*-CP-2 formulated with different adjuvants or alum adjuvant alone. Individual proportions are shown for each dog and the mean value for each group is denoted by a bar. Where the proportions were significantly different ($P < 0.1$ using a Wilcoxon–Signed Ranks test) between vaccine and control groups, *P* values are denoted beneath the mean.

Figure 55. Mean and medians of the worm burdens in vaccinated dogs relative to control (ASO3) dogs. 1 = Ac-ASP-2; 2 = Ac-API; 3 = Ac-MEP; 4 = Ac-APR-1; 5 = ASO3 (adjuvant).

Figure 56. Reduction in QECs (Quantitative Egg Counts) following vaccination and challenge.

Figure 57A-C. A, cDNA sequence of A. caninum GST (SEQ ID NO: 76) and B, corresponding amino acid sequence (SEQ ID NO: 77). C, alignment of coding region and amino acid sequence. Amino acids 1-19 are signal peptide.

Figure 58A and B. Graphic representation of adult worms recovered from the vaccinated and control dogs.

Figure 59. Median adult hookworm counts after use of trimmed mean calculation.

Figure 60. A, Median and B, mean reduction in quantitative egg counts.

Figure 61A and B. Na-CP-2: A, cDNA (SEQ ID NO: 83) and B, amino acid sequence (SEQ ID NO: 84).

Figure 62A and B. Na-CP-3: A, cDNA (SEQ ID NO: 85) and B, amino acid sequence (SEQ ID NO: 86).

Figure 63A and B. Na-CP-4: A, cDNA (SEQ ID NO: 87) and B, amino acid sequence (SEQ ID NO: 88).

Figure 64A and B. Na-CP-5: A, cDNA (SEQ ID NO: 89) and B, amino acid sequence (SEQ ID NO: 90).

Figure 65A and B. Na-MEP-1: A, cDNA (SEQ ID NO: 91) and B, amino acid sequence (SEQ ID NO: 92).

Figure 66A and B. Ac-16: A, cDNA (SEQ ID NO: 93) and B, amino acid sequence (SEQ ID NO: 94).

Figure 67A and B. Ay-16: A, cDNA (SEQ ID NO: 95) and B, amino acid sequence (SEQ ID NO: 96).

Figure 68A and B. Ac-CP-1: A, cDNA (SEQ ID NO: 97) and B, amino acid sequence (SEQ ID NO: 98).

Figure 69A and B. Ac-Cys: A, cDNA (SEQ ID NO: 99) and B, amino acid sequence (SEQ ID NO: 100).

Figure 70A and B. Ac-MEP-2: A, cDNA (SEQ ID NO: 101) and B, amino acid sequence (SEQ ID NO: 102).

Figure 71A and B. Ac-TTR-2: A, cDNA (SEQ ID NO: 103) and B, amino acid sequence (SEQ ID NO: 104).

Figure 72A and B. Ay-APR-1: A, cDNA (SEQ ID NO: 105) and B, amino acid sequence (SEQ ID NO: 106).

Figure 73A and B. Ay-CYS: A, cDNA (SEQ ID NO: 107) and B, amino acid sequence (SEQ ID NO: 108).

Figure 74A and B. Na-16: A, cDNA (SEQ ID NO: 109) and B, amino acid sequence (SEQ ID NO: 110).

Figure 75A and B. Na-MTP-1: A, cDNA (SEQ ID NO: 111) and B, amino acid sequence (SEQ ID NO: 112).

Figure 76A and B. Na-103 (SAA-1): A, cDNA (SEQ ID NO: 113) and B, amino acid sequence (SEQ ID NO: 114).

Figure 77A and B. A, Geometric mean of antibody titers; B, reduction in worm burdens.

Figure 78A and B. A, EPG per group, the average of two cages per group of 10 hamsters. B, Percentage of change of Hb at necropsy relative to pre-challenge values.

Figure 79A and B. A, spleen weights of hamsters by group; B, percentage body weight change at necropsy relative to pre-challenge.

Figure 80A and B. geometric mean of IgG titers. Relationship between antibody titers and A, worm burden and B, QECs.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

The present invention provides compositions for use in eliciting an immune response to hookworm in a mammal. Such compositions may be utilized as vaccines for use in the treatment and/or prevention of hookworm infection. The vaccines comprise purified preparations of antigens which are derived from hookworm, and a pharmacologically acceptable carrier. By "derived from" we mean that the antigen is a biomolecule that originated from (i.e. was isolated from) a hookworm. For example, the antigen may be a protein, a polypeptide, or an antigenic fragment of a protein, or polypeptide, which constitutes part of a hookworm organism. Typically, such an antigen is isolated and at least partially purified from a hookworm by methods which are well known to those of skill in the art (for example, see Examples section below). When manufactured for use in eliciting an immune response or as a vaccine, such antigens may be "synthetic" i.e. obtained synthetically (e.g. by peptide synthesis

in the case of polypeptides and protein fragments), or "recombinant" i.e. obtained by genetic engineering techniques (e.g. by production in a host cell which harbors a vector containing DNA which encodes the antigen). Those of skill in the art will recognize that many such suitable expression systems are available, including but not limited to those which employ *E. coli*, yeast (e.g. *Pichia pastoris*), baculovirus/insect cells, plant cells, and mammalian cells, and. In preferred embodiments of the invention, the antigens are expressed in a yeast or baculovirus/insect cell expression system.

Examples of specific antigens, their amino acid primary sequences, and nucleic acid sequences which encode them are given herein. For ease of reference, Table I lists some exemplary antigens and their corresponding SEQ ID NOS. However, those of skill in the art will recognize that many variants of the sequences presented herein may exist or be constructed which would also function as antigens in the practice of the present invention. For example, with respect to amino acid sequences, variants may exist or be constructed which display: conservative amino acid substitutions; non-conservative amino acid substitutions; truncation by, for example, deletion of amino acids at the amino or carboxy terminus, or internally within the molecule; or by addition of amino acids at the amino or carboxy terminus, or internally within the molecule (e.g. the addition of a histidine tag for purposes of facilitating protein isolation, the substitution of residues to alter solubility properties, the replacement of residues which comprise protease cleavage sites to eliminate cleavage and increase stability, the addition or elimination of glycosylation sites, and the like, or for any other reason). Such variants may be naturally occurring (e.g. as a result of natural variations between species or between individuals); or they may be purposefully introduced (e.g. in a laboratory setting using genetic engineering techniques). All such variants of the sequences disclosed herein are intended to be encompassed by the teaching of the present invention, provided the variant antigen displays sufficient identity to the described sequences. Preferably, identity will be in the range of about 50 to 100%, and more preferably in the range of about 75 to 100%, and most preferably in the range of about 80 to 100% of the disclosed sequences. The identity is with reference to the portion of the amino acid sequence that corresponds to the original antigen sequence, i.e. not including additional elements that might be added, such as those described below for chimeric antigens.

TABLE I. Hookworm antigens, description, and corresponding SEQ ID NOS.

Source	Antigen	Description	SEQ ID NOs. /.	
			cDNA (Accession No.)	open reading frame (Accession No.)
Necator americanus				
	Na-ASP-1	secreted protein	SEQ ID NO: 1 (AF079521)	SEQ ID NO: 2 (AAD13340)
	Na-ASP-2	secreted protein	SEQ ID NO: 82 (AY288089)	SEQ ID NO: 69 (AAP41952)
	Na-ACE	cholinesterase	SEQ ID NO: 3 (AF36813)	SEQ ID NO: 4 (AAN05636)
	Na-CTL	C-lectin	SEQ ID NO: 5	SEQ ID NO: 6
	Na-APR-1	aspartic protease	SEQ ID NO: 7	SEQ ID NO: 8
	Na-APR-2	aspartic protease	SEQ ID NO: 9	SEQ ID NO: 10
	Na-CP-2	cysteine protease	SEQ ID NO: 83	SEQ ID NO: 84
	Na-CP-3	cysteine protease	SEQ ID NO: 85	SEQ ID NO: 86
	Na-CP-4	cysteine protease	SEQ ID NO: 87	SEQ ID NO: 88
	Na-CP-5	cysteine protease	SEQ ID NO: 89	SEQ ID NO: 90
	Na-MEP-1	metallo-endopeptidase	SEQ ID NO: 91	SEQ ID NO: 92
	Na-MTP-1	astacin protease	SEQ ID NO:111	SEQ ID NO: 112
	Na-103 (SAA-1)	surface protein	SEQ ID NO:113	SEQ ID NO:114

	Na-16	surface-associated antigen	SEQ ID NO:109	SEQ ID NO:110
Ancylostoma duodenale				
	Ad-ASP-1	secreted protein	(AF077402)	SEQ ID NO: 67 (AAD13339)
	Ad-ASP-2	secreted protein	(AY288088)	SEQ ID NO: 68 (AAP41951)
Ancylostoma caninum				
	Ac-TMP	met protease inhibitor	SEQ ID NO: 11 (AF372651)	SEQ ID NO: 12 (AAK58952)
	Ac-MEP-1	metallo-endopeptidase	SEQ ID NO: 13 (AF273084)	SEQ ID NO: 14 (AAG29103)
	Ac-MEP-2	metallo-endopeptidase	SEQ ID NO:101	SEQ ID NO:102
	Ac-MTP-1	astacin protease	SEQ ID NO: 15 (AY036056)	SEQ ID NO: 16 (AAK62032)
	Ac-ASP-1	secreted protein	SEQ ID NO: 17 (AF132291)	SEQ ID NO: 18 (AAD31839)
	Ac-ASP-2	secreted protein	SEQ ID NO: 19 (AF089728)	SEQ ID NO: 20 (AAC35986)
	Ac-ASP-3	secreted protein	SEQ ID NO: 21 (AY217004)	SEQ ID NO: 22 (AA063575)
	Ac-ASP-4	secreted protein	SEQ ID NO: 23 (AY217005)	SEQ ID NO: 24 (AA063576)

	Ac-ASP-5	secreted protein	SEQ ID NO: 25 (AY217006)	SEQ ID NO: 26 (AA063577)
	Ac-ASP-6	secreted protein	SEQ ID NO: 27 (AY217007)	SEQ ID NO: 28 (AA063578)
	Ac-TTR-1	transthyretin	SEQ ID NO: 29	SEQ ID NO: 30
	Ac-TTR-2	transthyretin	SEQ ID NO:103	SEQ ID NO:104
	Ac-103 (SAA-1)	surface protein	SEQ ID NO: 31 (AY462062)	SEQ ID NO: 32 (AAR25200)
	Ac-VWF	surface lectin	SEQ ID NO: 33	SEQ ID NO: 34
	Ac-CTL	C-lectin	SEQ ID NO: 35	SEQ ID NO: 36
	Ac-API	aspartyl protease inhibitor	SEQ ID NO :37	SEQ ID NO: 38
	Ac-MTP-1	astacin protease	SEQ ID NO: 39	SEQ ID NO: 40
	Ac-MTP-2	astacin protease	SEQ ID NO: 41	SEQ ID NO: 42
	Ac-MTP-3	astacin protease	SEQ ID NO: 43	SEQ ID NO: 44
	Ac-FAR-1	retinol binding	SEQ ID NO: 45	SEQ ID NO: 46
	Ac-KPI-1	protease inhibitor	SEQ ID NO: 47	SEQ ID NO: 48
	Ac-APR-1	aspartic protease	SEQ ID NO: 49	SEQ ID NO: 50
	Ac-APR-2	pepsinogen	SEQ ID NO: 51	SEQ ID NO: 52
	Ac-AP	anticoagulant	SEQ ID NO: 53	SEQ ID NO: 54
	Ac-CP-1	cysteine protease	SEQ ID NO: 97	SEQ ID NO: 98
	Ac-CP-2	cysteine protease	(U18912)	(AAC46878)
	Ac-CYS	cystatin	SEQ ID NO: 99	SEQ ID NO: 100

	Ac-GST	glutathione S transferase	SEQ ID NO: 76	SEQ ID NO: 77
	Ac-16	surface-associated antigen	SEQ ID NO: 93	SEQ ID NO: 94
Ancyclostoma ceylanicum				
	Ay-ASP-1	secreted protein	SEQ ID NO: 55 (AY136548)	SEQ ID NO: 56 (AAN11402)
	Ay-ASP-2	secreted protein	SEQ ID NO: 57 (AY288090)	SEQ ID NO: 58 (AAP41953)
	Ay-MTP-1	astacin protease	SEQ ID NO: 59 (AY136547)	SEQ ID NO: 60 (AAN11401)
	Ay-API-1	aspartyl protease inhibitor	SEQ ID NO: 61	SEQ ID NO: 62
	Ay-TTR	transthyretin-like	SEQ ID NO: 63	SEQ ID NO: 64
	Ay-16	surface-associated antigen	SEQ ID NO: 95	SEQ ID NO: 96
	Ay-APR-1	aspartic protease	SEQ ID NO:105	SEQ ID NO:106
	Ay-CP-2	cysteine protease	(AF522068)	(AAM82155)
	Ay-CYS	cystatin	SEQ ID NO:107	SEQ ID NO:108

The invention also encompasses chimeric antigens, for example, antigens comprised of the presently described amino acid sequences plus additional sequences which were not necessarily associated with the disclosed sequences when isolated but the addition of which conveys some additional benefit. For example, such benefit may be utility in isolation and purification of the protein, (e.g. histidine tag, GST, and maltose binding protein); in directing

the protein to a particular intracellular location (e.g. yeast secretory protein); in increasing the antigenicity of the protein (e.g. KHL, haptens). All such chimeric constructs are intended to be encompassed by the present invention, provided the portion of the construct that is based on the sequences disclosed herein is present in at least the indicated level of homology.

Those of skill in the art will recognize that it may not be necessary to utilize the entire primary sequence of a protein or polypeptide in order to elicit an adequate antigenic response to the parasite from which the antigen originates. In some cases, a fragment of the protein is adequate to confer immunization. Thus, the present invention also encompasses antigenic fragments of the sequences disclosed herein, and their use in vaccine preparations. In general, such a fragment will be at least about 10-13 amino acids in length. Those of skill in the art will recognize that suitable sequences are often hydrophilic in nature, and are frequently surface accessible.

Likewise, with respect to the nucleic acid sequences disclosed herein, those of skill in the art will recognize that many variants of the sequences may exist or be constructed which would still function to provide the encoded antigens or desired portions thereof. For example, due to the redundancy of the genetic code, more than one codon may be used to code for an amino acid. Further, as described above, changes in the primary sequence of the antigen may be desired, and this would necessitate changes in the encoding nucleic acid sequences. In addition, those of skill in the art will recognize that many variations of the nucleic acid sequences may be constructed for purposes related to cloning strategy, (e.g. for ease of manipulation of a sequence for insertion into a vector, such as the introduction of restriction enzyme cleavage sites, etc.), for purposes of modifying transcription (e.g. the introduction of promoter or enhancer sequences, and the like), or for any other suitable purpose. All such variants of the nucleic acid sequences disclosed herein are intended to be encompassed by the present invention, provided the sequences display about 50 to 100% identity to the original sequence and preferably, about 75 to 100% identity, and most preferably about 80 to100% identity. The identity is with reference to the portion of the nucleic acid sequence that corresponds to the original sequence, and is not intended to cover additional elements such as promoters, vector-derived sequences, restriction enzyme cleavage sites, etc. derived from other sources.

The antigens of the present invention may be derived from any species of hookworm, examples of which include but are not limited to *Necator americanus*, *Ancylostoma caninum*, *Ancylostoma ceylanicum* and *Ancylostoma duodenale*.

Examples of suitable hookworm antigens include but are not limited to Na-ASP-1, Na-ACE, Na-CTL, Na-APR-1, NA-APR-2, Ac-TMP, Ac-MEP-1, Ac-MTP-1, Ac-ASP-1, Ac-ASP-2, Ac-ASP-3, Ac-ASP-4, Ac-ASP-5, Ac-ASP-6, Ac-TTR-1, Ac-103, Ac-VWF, Ac-CTL, Ac-API, Ac-MTP-1, Ac-MTP-2, Ac-MTP-3, Ac-FAR-1, Ac-KPI-1, Ac-APR-1, Ac-APR-2, Ac-AP, Ay-ASP-1, Ay-ASP-2, Ay-MTP-1, Ay-API, and Ay-TTR.

In some embodiments of the invention, the antigenic entity is an activation associated secretory protein, examples of which include but are not limited to Na-ASP-1, Ac-ASP-3, Ac-ASP-4, Ac-ASP-5, Ac-ASP-6, Ay-ASP-1, and Ay-ASP-2.

In other embodiments of the invention, the antigenic moiety is a protease, examples of which include but are not limited to metalloproteases (e.g. Ac-MTP-2, Ac-MTP-3; cysteine proteases; aspartic proteases (e.g. Ac-APR-1 and Ac-APR-2); and serine proteases.

In yet other embodiments of the invention, the antigen may be a lectin (e.g. Na-CTL, Ac-CTL).

In other embodiments of the invention, the antigen may be a protease inhibitor (e.g. Ac-API-1, Ay-API-1, Ac-AP, Ac-KPI-1).

In a preferred embodiment, the antigen utilized in the practice of the present invention is Ac-TMP, the DNA encoding sequence of which is given in Figure 6A (SEQ ID NO: 11), and the amino acid sequence of which is given in Figure 6B (SEQ ID NO: 12).

In another preferred embodiment, the antigen utilized in the practice of the present invention is Ac-MEP-1, the DNA encoding sequence of which is given in Figure 7A (SEQ ID NO: 13, and the amino acid sequence of which is given in Figure 7B (SEQ ID NO: 14).

In another preferred embodiment, the antigen utilized in the practice of the present invention is Ac-MTP-1, the DNA encoding sequence of which is given in Figure 8A (SEQ ID NO: 15, and the amino acid sequence of which is given in Figure 8B (SEQ ID NO: 16).

Other preferred antigens include but are not limited to *Na*-CTL (SEQ ID NOS. 5-6); *Na*-APR-1 (SEQ ID NOS. 7-8); *Na*-APR-2 (SEQ ID NOS. 9-10); *Ac*-TMP (SEQ ID NOS. 11-12); *Ac*-ASP-3 (SEQ ID NOS. 21-22); *Ac*-ASP-4 (SEQ ID NOS. 23-24); *Ac*-ASP-5 (SEQ ID NOS. 25-26); *Ac*-ASP-6 (SEQ ID NOS. 27-28); *Ac*-TTR-1 (SEQ ID NOS. 29-30); *Ac*-TTR-2

(SEQ ID NOS. 103-104) *Ac*-103 (SAA-1) (SEQ ID NOS. 31-32); *Ac*-VWF (SEQ ID NOS. 33-34); *Ac*-CTL (SEQ ID NOS. 35-36); *Ac*-API-1 (SEQ ID NOS. 37-38); *Ac*-MTP-1 (SEQ ID NOS. 39-40); *Ac*-MTP-2 (SEQ ID NOS. 41-42); *Ac*-MTP-3 (SEQ ID NOS: 43-44); *Ac*-KPI-1 (SEQ ID NOS: 47-48); *Ac*-APR-1 (49-50); *Ac*-APR-2 (SEQ ID NOS: 51-52); *Ay*-ASP-1 (SEQ ID NOS: 55-56); *Ay*-ASP-2 (SEQ ID NOS: 57-58); *Ay*-MTP-1 (SEQ ID NOS: 59-60); *Ay*-API-1 (SEQ ID NOS: 61-62); *Ay*-TTR (SEQ ID NOS: 63-64); *Na*-ACE (SEQ ID NOS: 3 and 4); *Na*-ASP-1 (SEQ ID NOS: 1 and 2); *Ac*-MEP-1 (SEQ ID NOS: 13-14);

Other preferred antigens for use in the practice of the present invention include *Ad*-ASP-1 (protein, SEQ ID NO: 67); *Ad*-ASP-2 (protein, SEQ ID NO: 68); *Na*-ASP-2 (protein, SEQ ID NO: 69; nucleotide, SEQ ID NO: 82); CP-2 antigens, e.g. Ac-CP-2 (Genebank Accession # U18912); *Na*-CP-2 (SEQ ID NOS: 83-84); *Na*-CP-3 (SEQ ID NOS: 85-86); *Na*-CP-4 (SEQ ID NOS: 87-88); *Na*-CP-5 (SEQ ID NOS: 89-90); *Ac*-CP-1 (SEQ ID NOS: 97-98); *Ac*-CP-2; *Ay*-CP-2; GST antigens, e.g. *Ac*-GST (protein SEQ ID NO: 77, nucleotide SEQ ID NO: 76); *Na*-MEP-1 (SEQ ID NOS: 91-92); *Na*-MTP-1 (SEQ ID NOS: 111-112); *Na*-103 (SAA-1) (SEQ ID NOS: 113-114); *Na*-16 (SEQ ID NOS: 109-110; *Ac*-MEP-2 (SEQ ID NOS: 101-102); *Ac*-CYS (SEQ ID NOS: 99-100); Ay-CYS (SEQ ID NOS: 107-108); *Ac*-16 (SEQ ID NOS: 93-94); *Ay*-16 (SEQ ID NOS: 95-96); Ay-APR-1 (SEQ ID NOS: 105-106).

The present invention provides compositions for use in eliciting an immune response which may be utilized as a vaccine against hookworm. By "eliciting an immune response" we mean that an antigen stimulates synthesis of specific antibodies at a titer of about >1 to about 1 x 10^6 or greater. Preferably, the titer is from about 10,000 to about 1 x 10^6 or more, and most preferably, the titer is greater than 1 x 10^6, and/or cellular proliferation as measured by, for example, ^{3}H thymidine incorporation. By "vaccine" we mean an antigen that elicits an immune response that results in a decrease in hookworm burden of a least about 30% in an organism in relation to a non-vaccinated (e.g. adjuvant alone) control organism. Preferably, the level of the decrease is about 50%, and most preferably, about 60 to about 70% or greater.

The present invention provides compositions for use in eliciting an immune response which may be utilized as a vaccine against hookworm. The compositions include a substantially purified hookworm antigen or variant thereof as described herein, and a pharmacologically suitable carrier. The preparation of such compositions for use as vaccines is well known to those of skill in the art. Typically, such compositions are prepared either as liquid solutions or

suspensions, however solid forms such as tablets, pills, powders and the like are also contemplated. Solid forms suitable for solution in, or suspension in, liquids prior to administration may also be prepared. The preparation may also be emulsified. The active ingredients may be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredients. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol and the like, or combinations thereof. In addition, the composition may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and the like. In addition, the composition may contain other adjuvants. If it is desired to administer an oral form of the composition, various thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders and the like may be added. The composition of the present invention may contain any such additional ingredients so as to provide the composition in a form suitable for administration. The final amount of hookworm antigen in the formulations may vary. However, in general, the amount in the formulations will be from about 1-99%.

The vaccine preparations of the present invention may further comprise an adjuvant, suitable examples of which include but are not limited to Seppic, Quil A, Alhydrogel, etc.

The preparations of the present invention may contain a single hookworm antigen. Alternatively, more than one hookworm antigen may be utilized in a preparation, i.e. the preparations may comprise a "cocktail" of antigens. In a preferred embodiment, such a cocktail will contain two or more antigens, and will be a combination of a larval stage antigen and an adult stage antigen. Examples of suitable larval stage antigens include but are not limited to ASP-2, MTP-1, 103 (SAA-1), 16 and GST. Examples of suitable adult stage antigens include but are not limited to APR-1, CP-2, GST, MEP-1, APR-2, and TMP. GST is an antigen that is present at both the larval and adult stage. The antigens utilized in the cocktail may be from any species. However, in preferred embodiments of the invention, the antigens will be antigens derived from a human hookworm source such as *Na*-Asp-2, *Na*-APR-1, *Na*-CP-2, *Na*-GST, *Na*-MEP-1, *Ad*-Asp-2, *Ad*-APR-1, *Ad*-CP-2, *Ad*-GST, *Ad*-MEP-1, *Na*-MTp-1, *Ad*-MTP-1, *Na*-103 (*Na*-SAA), *Ad*-103 (*Ad*-SAA-1), *Na*-16, and *Ad*-16. Preferably, the cocktail will contain at least two antigens derived from a human hookworm source, at least one larval stage and at least one adult stage, such as, for example, either Na- or Ad- : Asp-2 APR-1; Asp-2 and CP-2; Asp-2 and GST; Asp-2 and MEP-1.

The present invention also provides a method of eliciting an immune response to hookworm and methods of vaccinating a mammal against hookworm. By eliciting an immune response, we mean that administration of the antigen causes the synthesis of specific antibodies (at a titer in the range of 1 to 1 x 10^6, preferably 1 x 10^3, more preferable in the range of about 1 x 10^3 to about 1 x 10^6, and most preferably greater than 1 x 10^6) and/or cellular proliferation, as measured, e.g. by ^3H thymidine incorporation. The methods involve administering a composition comprising a hookworm antigen in a pharmacologically acceptable carrier to a mammal. The vaccine preparations of the present invention may be administered by any of the many suitable means which are well known to those of skill in the art, including bu not limited to by injection, orally, intranasally, by ingestion of a food product containing the antigen, etc. In preferred embodiments, the mode of administration is subcutaneous or intramuscular.

The present invention provides methods to elicit an immune response to hook worn and to vaccinate against hookworm in mammals. In one embodiment, the mammal is a human. However, those of skill in the art will recognize that other mammals exist for which it would also be of benefit to vaccinate against hookworm, i.e. the preparations may also be used for veterinary purposes. Examples include but are not limited to companion "pets" such as dogs, cats, etc.; food source, work and recreational animals such as cattle, horses, oxen, sheep, pigs, goats, and the like.

Those of skill in the art will recognize that, in general, in order to vaccinate (or elicit an immune response in) a species of interest (e.g. humans) against hookworm, the antigen which is utilized will be derived from a species of hookworm which parasitizes the species of interest. For example, in general, antigens from *Necator americanus* may be preferred for the immunization of humans, and antigens from *Ancylostoma caninum* may be preferred for the immunization of dogs. However, this may not always be the case. For example, *Ancylostoma caninum* is known to parasitize humans as well as its primary canine host. Further, cross-species hookworm antigens may sometimes be highly effective in eliciting an immune response in a non-host animal, i.e. in an animal that does not typically serve as host for the parasite from which the antigen is derived. Rather, the measure of an antigen's suitability for use in an immune-stimulating or vaccine preparation is dependent on its ability to confer protection against invasion and parasitization by the parasite as evidenced by, for example, hookworm burden reduction or inhibition of hookworm associated blood loss (e.g. as measured by

hematocrit and/or hemoglobin concentration. For example, for use in a vaccine preparation, an antigen upon administration results in a reduction in worm burden of at least about 30%, preferably at least about 50%, and most preferably about 60 to about 70%.

In one embodiment of the present invention, a method for enabling vaccination of a patient against infectious diseases is provided. The method involves chemically treating hookworm infection to a degree sufficient to increase lymphocyte proliferation, followed by vaccinating the patient against said infectious disease. The method is based on evidence provided in Example 10 which shows that hookworm infestation causes anergy to hookworm and possibly other antigen stimulation. Therefore, by chemically treating hookworm infected patients prior to vaccination against hookworm or any infectious agent, the response to the vaccination will be improved. Examples of infectious diseases against which vaccination outcomes may be improved include but are not limited to HIV, tuberculosis, malaria, and routine childhood vaccinations (e.g. measles, tetanus, diphtheria, pertussis, polio, and the like).

Examples of agents with which hookworm may be chemically treated include but are not limited to albendazole and other antihelminthic drugs.

Certain of the antigens described herein may also be useful in the vaccination against other parasites, for example (including but not limited to) *Schistosoma sp* and soil transmitted parasites such as *Ascaris sp* and *Trichuris sp.* This may be due to the potential cross reactivity between the hookworm antigens and antigens from these species.

Certain of the antigens described herein may also be useful in the treatment of other neoplastic, autoimmune, and cardiovascular conditions, as well as for the treatment of pro-inflammatory states. Such uses of other hookworm antigens have been described in, for example, United States patent 5,427,937 to Capello et al. and United States patent 5,753,787 to Hawdon.

The present invention also provides the following nucleic acid and amino acid sequences: SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID

NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, and SEQ ID NO: 64. The sequences represent cDNA sequences and the amino acid sequences (open reading frames) which they encode. While the sequences themselves are being claimed, other sequences with a high level of identity in comparison to those described are also contemplated, e.g. sequences having at least about 65 to100% identity, or preferably about 75 to 100% identity, or most preferably at least about 80 to 100% identity, to the sequences that are given.

In particular, the sequences for Ac-APR-2 (SEQ ID NOS: 51 and 52) and Ay-TTR-1 (SEQ ID NOS: 63 and 64) are partial sequences which represent the majority of the antigen sequence. Thus, the present invention encompasses the entire Ac-APR-2 antigen and the entire Ay-TTR-1 antigen.

Further, those of skill in the art will recognize that the Ay-TTR-1 and Ay-TTR-2 antigens which are provided in the present application are representative of the Ay-TTR family of antigens present in many species of nematodes. As such, an Ay-TTR antigen from any nematode is intended to be encompassed by the present invention. In particular, any Ay-TTR antigen derived from a hookworm species including but not limited to *Necator americanus*, *Ancylostoma caninum*, *Ancylostoma ceylanicum*, and *Ancylostoma duodenale*, are encompassed.

Additional sequences that are provided by the present invention include: SEQ ID NO: 76 and SEQ ID NO: 77, representing Ac-GST cDNA and corresponding amino acid sequence, respectively; SEQ ID NO: 83 and SEQ ID NO: 84, representing Na-CP-2 cDNA and corresponding amino acid sequence, respectively; SEQ ID NO: 85 and SEQ ID NO: 86, representing Na-CP-3 cDNA and corresponding amino acid sequence, respectively; SEQ ID NO: 87 and SEQ ID NO: 88, representing Na-CP-4 cDNA and corresponding amino acid sequence, respectively; SEQ ID NO: 89 and SEQ ID NO: 90, representing Na-CP-5 cDNA and corresponding amino acid sequence, respectively; SEQ ID NO: 91 and SEQ ID NO: 92, representing Na-MEP-1 cDNA and corresponding amino acid sequence, respectively; SEQ ID NO: 93 and SEQ ID NO: 94, representing Ac-16 cDNA and corresponding amino acid sequence, respectively; : SEQ ID NO: 95 and SEQ ID NO: 96, representing Ay-16 cDNA and

corresponding amino acid sequence, respectively; SEQ ID NO: 97 and SEQ ID NO: 98, representing Ac-CP-1 cDNA and corresponding amino acid sequence, respectively; SEQ ID NO: 99 and SEQ ID NO: 100, representing Ac-CYS cDNA and corresponding amino acid sequence, respectively; SEQ ID NO: 101 and SEQ ID NO: 102, representing Ac-MEP-2 cDNA and corresponding amino acid sequence, respectively; SEQ ID NO: 103 and SEQ ID NO: 104, representing Ac-TTR-2 cDNA and corresponding amino acid sequence, respectively; SEQ ID NO: 105 and SEQ ID NO: 106, representing Ay-APR-1 cDNA and corresponding amino acid sequence, respectively; SEQ ID NO: 107 and SEQ ID NO: 108, representing Ay-Cys cDNA and corresponding amino acid sequence, respectively; SEQ ID NO: 109 and SEQ ID NO: 110, representing Na-16 cDNA and corresponding amino acid sequence, respectively; SEQ ID NO: 111 and SEQ ID NO: 112, representing Na-MTP-1 cDNA and corresponding amino acid sequence, respectively; and SEQ ID NO: 113 and SEQ ID NO: 114, representing Na-SAA-1 cDNA and corresponding amino acid sequence, respectively.

EXAMPLES

Example 1. Molecular Cloning and Purification of Ac-TMP

Materials and Methods.

Immunoscreening of adult A. caninum library Preparation of anti- *A. caninum* secretory product antibody. One hundred living adult stage *Ancylostoma caninum* hookworms were recovered from the intestines of an infected dog, at necropsy (6 weeks post-infection), as described previously (Hotez and Cerami, 1983). The adult worms were washed three times in sterile PBS, then maintained in 15 ml RPM 1640 containing 25 mM HEPES, 100 units/ml of ampicillin and 100 μg/ml streptomycin at 37C (5% CO_2) for 24 hours. The supernatant was collected, concentrated with PEG6000, and dialyzed against 1 L phosphate buffered saline (pH 7.2) overnight at 4 °C. Following dialysis, the secreted products were centrifuged at 10,000xg for 10 min, and the supernatant was recovered.

A rabbit was immunized by subcutaneous injection with the hookworm-secreted proteins (400 ug) emulsified with complete Freund's adjuvant. Subsequently, the rabbit was immunized at two week intervals with the same quantity of hookworm secreted proteins emulsified with incomplete Freund's adjuvant for a total of three immunizations. The final

bleed was obtained 10 days after the final immunizations, and the serum was separated from whole blood and stored at -20 °C.

Construction of the cDNA expression ZapII (Stratagene, La Jolla CA) library was reported previously (Capello et al., 1996)). An estimated 5 x 10^5 plaques were screened with the rabbit anti-*A. caninum* adult secretory product antibody according to manufacturer's instructions. Briefly, 5 x 10^4 plaques were plated on an LB agar plate. *A. caninum* antigen expression was induced by covering the plaques with nitrocellulose membranes soaked with 10 mM IPTG. Four hours after incubation at 37 °C, the membranes were lifted, blocked with 5% non-fat milk in PBS, and then incubated with the rabbit antibody (1:500 dilution) for 1 hour at 24 °C. The membranes were washed three times with PBS buffer containing 0.1% Tween-20 (PBS-Tween) and then incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (Sigma) at a 1:1000 dilution at 24 °C for another hour. The membranes were washed again three times with PBS-Tween and then developed with 3,3'-diaminobenzidine (DAB) substrate and hydrogen peroxide. The putative positive clones were scored and isolated for secondary screening.

The immunopositive clones were excised into pBluscript phage according to manufacturer's instructions (Stratagene), Phagemid DNA was extracted using the alkaline lysis method (Qiagen) and double strand sequencing was performed using flanking vector primers (T_3 and T_7). Nucleotide and deduced amino acid sequences were compared to existing sequences in GenBank by BLAST searching. ESEE 3.1 software was used for sequence analysis.

Reverse transcription polymerase chain reaction (RT-PCR) amplification.

RT-PCR was used to determine the developmental stage specificity of *Ac-tmp* mRNA transcription. *A. caninum* eggs, L I and L2 larval stages, and L3 infective larvae were obtained as described previously (Hawdon et al, 1999). The total RNA was isolated from each life history stage using TRIzol reagent (GIBCO BRL). Single-strand cDNA was synthesized using oligo d(T) primer and MMLV-RT(GIBCO BRL). Specific primers (TIMP3'-1HR and TIMP5'-2ER based on the sequence of *Ac-tmp* from 60bp to 440bp were used to amplify the *Ac-tmp* cDNA. PCR reaction parameters were comprised of 94 °C denaturing for 1 min, 55 °C annealing for 1 min, 72C extension for 2 min. A total 30 cycles were performed.

Purification of Ac-TMP natural product. Optimization of semi-preparative reverse phase chromatographic conditions for the fractionations of *A. caninum* adult secretory products was carried out on a 510 HPLC system (Waters), equipped with a 490 E multiwavelength detector with a semi-preparative flow-cell, set at 214, 280, 260 and 254mm and a 250mm x 4.6 I.D. YMC-Pack Protein-RP, 200Å, 5μm C_4 Column (Waters). The adult *A. caninum* secretory products used as starting material were collected over 15 hr from 1260 adult hookworms in 15 ml RPMI 1640 containing 25 mM HEPES, 100 units/ml ampicillin, 100 μg/ml streptomycin and 100 μg/ml gentamicin at 37 °C. The supernatant was concentrated by ultrafiltration in a Centricon-3 microconcentrator (Amicon) to 0.3 vol. before centrifugation for 1 hr at 7,500 x g. Approximately 0.6 mg of the parasite secretory protein was chromatographed. Eluent A was 0.01% Trifluoroacetic acid (TFA) in water, and eluent B was 0.01% TFA in acetonitrile. A 40-min linear aradient from 0-80% B was run at a flow-rate of 1 ml/min. Fractions of 0.5 min were collected, lyophilized, and were used for further purification and analysis by SDS-PAGE (Laemmli, 1970). For SDS-PAGE, 2 μl of secretory products as well as the 10 μl of HPLC isolated fraction number 51 were mixed with the same volume of 2X SDS-PAGE sample buffer (4% SDS, 2.5% 2- mercapto ethanol, 15% glycerol) and boiled for 5 min. The samples were run on a 4-20% gradient SDS-PAGE gel at 100 V for 2 hours. The gel was stained with silver according to manufacturer's instruction (BIO-RAD).

RP-HPLC of Fraction 51, the fraction that contained the most abundant *A. caninum* secretory protein from the semi-preparative separation, was carried out on a 510 HPLC system equipped as described above using a 250 mm. x 3.0 I.D. YMC protein RP, 200 A, 5 μm C4 column. Eluent A was 0.01% TFA in water, and B was 0.01% TFA in acetonitrile. A 30-min linear gradient from 0-60% B was run at a flow-rate of 1 ml/min. Fractions of 0.5 min were collected and lyophilized. The major protein peak collected from this separation was subjected to amino acid sequence analysis and SDS-PAGE (Laemmli, 1970). Amino acid sequence analysis based on the Edman degradation of protein was performed on procise 494 model protein sequencer (Applied Biosystems) equipped with a 785A programmable detector and a 140C pump system, by ProSeq, Inc. (Boxford MA). The sequencer products were identified using standard procise 610A software.

To confirm that the N-terminal sequence corresponded to Ac-TMP, degenerate oligonucleotide primers were synthesized in both orientations that corresponding to the partial

N-terminal peptides sequence of fraction number 51. Paired flanking degenerate vector primers were used to amplify the product from DNA obtained from the adult cDNA library constructed in ZapII . The "hot start" PCR conditions were 10 mM Tris-HCI (pH 8.5) containing 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, and 1 μl cDNA library, in 20 μl reaction. The reactions were heated at 94 °C for 5 min, then lowered to 85 °C for 5 min, then 1 unit of Taq DNA polymerase (GIBCO BRL) was added. This was followed by 30 cycles of 1 min of denaturation at 94 °C, 1 min of annealing at 55 °C, and 2 min of extension at 72 °C. The PCR products were run on an agarose gel and stained with ethidium bromide. The PCR products were gel purified with the QIAEX II Gel Extraction kit (Qiagen, Valencia, CA), and sequenced.

Results for Example 1

Ac-TMP cDNA. Ac-TMP cDNA was cloned from an adult hookworm cDNA library by immunoscreening with rabbit antibody directed against whole *A.caninum* adult secretory products. Two positive identical clones were isolated. The full-length cDNA consists of 559 bps (SEQ ID NO: 11) encoding an open reading frame (ORF) of 140 amino acids (SEQ ID NO: 12) and a poly-A tail at the 3' end. The predicted ORF has a calculated molecular weight of 16,100 daltons and a theoretical pI of 7.55. There is a hydrophobic signal peptide sequence with a signal peptidase cleavage site between amino acids 16 and 17. Ac-TMP has a signature N terminal Cys-X-Cys sequence immediately following the signal peptide. One putative N linked glycosylation site (N-X-T) exists between amino acids 119 and 122 (Fig.6B).

GenBank database searching revealed that the predicted amino acid sequence of this molecule shares 33 percent identity and 50 percent similarity to the N-terminal domain of human tissue inhibitor of metalloproteinase 2 (TIMP-2). Both Ac-TMP and a putative TIMP from the free-living nematode *Caenorhabditis elegans* are comprised of a single domain and lack a second, C-terminal domain that is characteristic of vertebrate TIMPs (data not shown).

RT-PCR amplification. To identify the life-history stage specific expression of Ac-TMP, mRNAs were extracted from different developmental stages of *A.caninum* and reverse transcribed to cDNA with Ac-TMP specific primers. RT-PCR produced a 380 bp specific band that was only amplified from adult cDNA. No amplification was seen from the cDNA of eggs, Li-L2 and L3 life history stages. Amplification of *A. caninum* genomic DNA revealed two bands suggestive of a possible intron or the existence of a second, related Ac-TMP gene (data not shown).

Identification of Ac-TMP in secretory products of *A. caninum* adult worm. To confirm that Ac-TMP is released by adult *A. caninum* hook-worms, the protein was identified in and purified from parasite secretory products via RP-HPLC. Each of the major peaks were subjected to amino acid sequence analysis as part of a larger *A. caninum* proteomics study (data not shown). The peak of protein corresponding to "Fraction 51" was selected for further study and re-chromatographed. Fraction 51 was comprised of one predominant band after silver staining that migrated with an apparent molecular weight of Mr =16,000. The N-terminal peptide sequence (20 amino acids) of this fraction was an identical match with the sequence of the predicted ORF of Ac-TMP after the predicted signal peptidase cleavage site. Based on the calculated area under the curve of HPLC peak 51 relative to the total area of the entire secretory product profile, Ac-TMP was determined to comprise approximately 6.3 percent of the total *A. caninum* secretory products. This identified the molecule as one of the most abundant proteins released by adult *A. caninum*. The abundance of Ac-TMP in hookworm secretory products was confirmed by visual inspection on SDS-PAGE. Paired degenerate primers based on the sequence of the first seven amino acids were used to construct PCR products from the adult hookworm cDNA library. DNA sequence of the PCR products confirmed the identity to Ac-TMP cDNA (data not shown).

This example demonstrates that TMP is the most abundant protein secreted by hookworms and that the protein has been cloned and expressed, and the recombinant protein isolated.

Example 2. Molecular cloning and characterization of *Ac-mep*-1.

Materials and Methods.

Parasites. *A. caninum* parasites were maintained in beagles as described previously (Schad 1982). Third stage infective larvae (L3) were isolated from charcoal copro-cultures and stored in BU buffer (Hawdon et al. 1995). Adult *A. caninum* worms were collected from infected dogs upon necropsy. These worms were washed three times in PBS, snap frozen in liquid nitrogen, and stored at -80 □C.

Nucleic acids Genomic DNA was isolated from adult *A. caninum* by standard methods (Ausubel et al. 1993). *A. caninum* RNA was isolated by grinding previously frozen (-80 □C) adult worms in the presence of Trizol reagent (Gibco BRL) and following manufacturers

protocol. cDNA was prepared from RNA by the ProSTAR First Strand RTPCR Kit (Stratagene) according to the manufacturer's instructions.

A. caninum genomic and cDNA libraries An *A. caninum* genomic DNA library was constructed as follows: 30 ug *A. caninum* genomic DNA was partially digested (37 □C for 5 min) by 8 U *Sau*3A restriction enzyme (NEB) in a 100 ul volume with recommended buffer. The digested DNA was then ethanol precipitated and pelleted by standard methods. The resulting pellet was dried, dissolved in water, and ligated into the Lambda-FIXII vector (Stratagene) according to manufacture's protocol. This ligation reaction was then packaged with Gigapack Gold packaging extract (Stratagene) and amplified. An *A. caninum* adult cDNA library was constructed previously (Capello et al. 1996) in lambda ZAPII (Stratagene) vector.

Metalloprotease cloning Cloning the *Ac-mep*-1 cDNA began with PCR on adult hookworm library cDNA using a degenerate primer and oligo-dT. A degenerate primer was designed against a conserved sequence containing the zinc binding motif observed in an BLAST alignment of two hypothetical zinc metalloprotease genes from *C. elegans* (GenBank™ accession numbers T22668 and Q22523) The reaction conditions were as follows: 85 ng template DNA, 1X thermophillic DNA buffer (Promega), 2.5mM MgCl2, 0.2 mM dNTP's, 2 uM each primer, 1 U taq DNA polymerase (Promega), in 20 μl total volume. The reactions were cycled at 94 °C for 1 min, 55C for 1 min, and 72C for 1 min 35 times. This PCR yielded a fragment which when cloned (pGEM-T, Promega) and sequenced represented 458 bp (including 21 residues of the poly A tail) of the 3'*Ac-mep*-1 cDNA (Clone MP-1). Utilizing the MP-1 as the basis for specific primer design additional sequence of *Ac-mep*-1 (Clone MP-2) was identified by PCR on library DNA with T3 (vector) and MEP-R1 gene specific primers. Reactions were conducted on serial dilutions of library DNA until a unique product was amplified and then cloned. Reaction conditions were as described above.

In a similar clone MP-3 was amplified with T3 and MEP-R2 primers. The 5'-RACE kit from GibcoBRL was employed to identify the 5' end of *Ac-mep*-1. Briefly, first strand cDNA was produced in a reverse transcription reaction with the *Ac-mep*-1 specific primer RACE-R1 on freshly prepared RNA. This cDNA was then poly C tailed at its 3' end with terminal deoxytransferase and used as template in a PCR reaction with anchor primer AAP (GibcoBRL) and gene specific reverse primer MEP-R2. The resulting products were diluted and used as

template in a hemi-nested PCR reaction with anchor primer UAP (GibcoBRL) and gene specific primer MEP-R3. The PCR product generated was cloned and termed MP-4.

More 5' sequence was identified from a genomic DNA clone (G-MEP) of *Ac-mep*-1 like sequence. Multiple clones were sequenced to confirm the *Ac-mep*-1 cDNA and the full length coding region of *Ac-mep*-1 was PCR amplified (clone FL-1) under the conditions described above as a single fragment utilizing suitable primers.

Sequence analysis Alignment of the partial *Ac-mep*-1 clones was conducted using MEGALIGN software from DNASTAR Inc. (version 3.7.1). BLAST analysis of the initial sequences used for degenerate primer design and the predicted open reading frame (ORF) of *Ac-mep*-1 was conducted using the National Center for Biotechnology Information BLAST utility. Sequence analysis of *Ac- mep*-1 was conducted using the Curatools sequence analysis utility (Curagen Corp., New Haven, CT.). The FGENESH gene finder utility (CGG WEB server (genomic.sanger.ac.uk) with settings to analyze *C. elegans* DNA was utilized for gene predictions from the genomic DNA clone G-MEP. Identification of potential exon sequences in GMEP was accomplished with the Wise2 sequence analysis utility (sanger.ac.uk/Software Wise2/).

Northern blotting Northern blot analysis was conducted on Trizol (GibcoBRL) isolated total RNA from ten adult worms. This RNA was fractionated on a 1.2% formaldehyde gel and blotted to Hybond-*N* membrane (Amersham) by standard methods. The blot was probed with a ^{32}P random prime labeled DNA fragment representing bp 780–2688 of the *Ac-mep*-1 cDNA.

Developmental RT-PCR RT-PCR was used to investigate *Ac-mep*-1 transcription in *A. caninum* life history stages. For these reactions cDNA from egg, L1, non-activated and activated L3 and adult worms were tested with *Ac-mep*-1 specific primers MEP-F1 and MEP-R1. The quality if these cDNAs was verified in separate reactions using primers PKA-F and PKA-R, which are specific for *A. caninum* protein kinase A (Hawdon et al. 1995). The reaction conditions were identical to those defined in Section 2.4.

Anti-Ac-mep-1 antibody A cDNA fragment representing 610 amino acids from the C-terminal portion of Ac-MEP-1 was amplified from the adult *A. caninum* cDNA lambda library by PCR using suitable primers. This fragment was T/A cloned into pGEM (Promega) from which it was cloned into pET28c expression vector (Novagen) at the HindIII site by standard methods (Sambrook and Russell, 2001). Bacterial protein expression of truncated Ac-MEP-1 (tAc-MEP-

1) was induced by the addition of 1 mM IPTG to a culture of BL21(DE3)PlysS (Stratagene) cells transformed with the tAc-MEP-1/pET28c construct.

The expressed protein was insoluble. In order to purify t*Ac-mep*-1 the induced cell pellets were frozen (BL21(DE3)PlysS cells lyse after freezing), resuspended in one-tenth vol. of 50 mM tris pH 8.0, 2 μM EDTA, sonicated until no longer viscous and then centrifuged at 12, 000 xg for 15 min (Sorvall RC5B, GSA rotor). The resulting pellet was resuspended in 15 ml 1% SDS, 0.5% B-mercaptoethanol, sonicated, boiled for 5 min, and then incubated at room temperature for 2 h. Undissolved debris was removed by repeat centrifugation. The supernatant was dialyzed exhaustively against phosphate buffered saline (pH 7.4) to remove the BME. The protein was purified on HisBind (Novagen) nickel resin affinity column according to the manufacturer's protocol without denaturant. Groups of five male Balb/c mice (6-week-old) were immunized intraperitoneally with 20 ug of alum-precipitated tAc-MEP-1 or alum alone as control. The mice were subsequently boosted twice at 2-week intervals. One week after the third and final immunization, sera was collected, pooled, and used as a primary antibody in the western blot and immunostaining analysis.

Western blotting Proteins separated by 10% SDS-PAGE were transferred to methanol charged Immobilon-P PVDF membranes (Millipore) in transfer buffer (39 mM glycine, 48 mM tris base, 0.037% SDS, pH 8.3) for 18 h at 30V. The membrane was blocked in 5% nonfat milk in PBS (blocking buffer), for 1 h at room temperature (RT) with gentle shaking and incubated with *E. coli* absorbed primary mouse anti-tAc-MEP-1 antibody (1:1500) diluted in blocking buffer for 1 h at RT. The membrane was then washed three times in blocking buffer (10 min each), and incubated for 1 h at RT with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:5000) in blocking buffer with shaking. Finally, the membrane was washed three times in PBS for 15 min and developed with Renaissance (NEN Life Science Products) chemiluminescent reagents.

Immunolocalization Adult *A. caninum* worms were paraffin embedded and sectioned by standard methods. In situ immunolocalization of Ac-MEP-1 was accomplished by incubating de-parrafinized worm sections in a 1:100 dilution (in PBS, pH 7.4) of mouse anti-tAc-MEP-1 or control sera (see above) for 1 h at RT. The sections were washed three times in PBS and incubated in a 1:200 dilution of goat anti-mouse IgG at 25 °C for 1 h followed by washing in

PBS (three times). Sections were then visualized with a Olympus IX-50 inverted fluorescence microscope (U-MWIG filter) and photographed.

Results for Example 2

cDNA structure of *Ac-mep-* 1 The cloning strategy employed in obtaining the complete coding sequence of *Ac-mep*-1 was as follows: About 2.6 kb of the *Ac-mep*-1 transcript was identified by sequencing degenerate PCR clone MP-1, PCR derived clones MP-2, MP-3 and the 5' RACE clone MP-4. Although there was a methionine codon close to the 5' end of the RACE product, this codon was preceded by 58 in-frame amino acids that contained no stop, suggesting that MP-4 did not represent the actual 5' end of *Ac-mep*-1. In addition, we have been unable to obtain a cDNA clone (by PCR) that included a spliced leader sequence. Therefore, G-MEP, a genomic DNA clone of *Ac-mep-* 1 like sequence (98.7% exon identity), was examined with a gene prediction program for *C. elegans* DNA and a different potential transcription start site than was identified by 5' RACE was identified . This prediction extended 158 bp beyond the 5' RACE sequence and increased the deduced coding region by 91 amino acids. Utilizing this prediction the entire coding region of *Ac-mep*-1 was amplified as a single product of 2.7 kb product and the clone was confirmed by partially sequencing both its ends. The total length of the *Ac-mep*-1 transcript is ~2.8 kb as verified by Northern blot (non-coding portions of the 5' and 3' ends were not amplified in the full length PCR). The deduced amino acid sequence of this transcript encodes a single ORF of 870 amino acids with four potential *N*-linked glycosylation sites (predicted pI=5.5, m.w.=98.7 kDa). The *N*-terminal amino acids of Ac-MEP-1 comprise a hydrophobic signal peptide sequence with a predicted cleavage after residue 22 (see Figure 7B). Two signature zinc-binding motifs characteristic of the Endopeptidase 24.11 family of metalloproteases (Hooper, 1994) were identified.

Ac-mep-1 is 66% similar and 48% identical to a metalloprotease (*Hc*-MEP1b) from the related trichostrongyle blood feeding nematode *H. contortus*. It is also equally similar to a metalloprotease (T25B6.2) from the non-parasitic nematode *C. elegans* (Gen-Bank™ T28906). Fourteen cysteine residues are highly conserved between these three molecules. Two additional cysteines (only one is conserved) are present in both Ac-MEP-1 and *Hc*-MEP1b.

Northern blot and developmental analysis of *Ac-mep*-1 expression Northern blot analysis reveals a single mRNA transcript of approximately 2.8 kb in adult hookworm mRNA (not shown). RT-PCR was employed to investigate the developmental specificity of *Ac-mep*-1

transcription. Of the cDNAs tested it was possible to identify transcription only in the adult stage of the parasite and not in hookworm eggs, L1 or activated and non-activated L3 larvae. In contrast, positive control PCR conducted on the same cDNAs with primers specific for A. *caninum* protein kinase A revealed amplification from all template cDNAs. Thus, *Ac-mep*-1 appears to be expressed exclusively in adult worms.

Western blot analysis and immunolocalization of *Ac-mep*- 1 in adult worm sections By western blotting, the mouse anti-MEP-1 antiserum strongly recognizes adult *A. caninum* proteins of ~90 and 100 kDa. Immunohistochemical analysis of adult worm sections localizes Ac-MEP-1 to the microvillar surface of the hookworm gut. The antiserum reacts strongly to the gut microvilli in sections of adult worm as compared with sections incubated with control sera. Weaker staining in the tegument of the adult worm was also occasionally noted. Although the function of Ac-MEP-1 is not known, its location along the microvillar surface of the parasite gut would suggest that the enzyme is in direct contact with the blood meal, and may, therefore, have a role in nutrient digestion.

This example demonstrates that MEP-1 is an important enzyme which allows hookworms to digest blood, and therefore is an attractive vaccine target. The recombinant MEP-1 protein has been cloned and expressed.

Example 3. AC-MTP Antigen Studies

Infective third-stage Ancylostoma hookworm larvae (L3) release a zinc-dependent metalloprotease that migrates with an apparent molecular weight of 50 kDa (Hawdon et al 1995a). The enzyme is released specifically in response to stimuli that induce feeding and development in the L3 (Hawdon et al, 1995b), and probably functions either in parasite skin and tissue invasion or ecdysis (Hotez et al, 1990). Because of its role in parasite-derived tissue invasion and molting, an anti-enzyme antibody response directed against Ac-MTP-1 might block larval migrations and parasite entry into the intestine. Ac-MTP- I is stage specific, and released by hookworm L3activated under hostlike conditions to resume feeding in vitro. Release of Ac-MTP- I during activation makes this molecule an attractive vaccine target.

Example 3A. Isolation of a cDNA from an *A. caninum* L3expression library that encodes a zinc-metalloprotease (Ac-mtp-1) of the astacin family.

Material and Methods

Antisera: Sera used for immunoscreening of the *A. caninum* L3 expression library were collected from 5 residents of Nanlin county in Anhui Province, China, under an IRB-approved human investigations protocol. *Ancylostoma duodenale* is the predominant hookworm in this region, with a ratio of *A. duodenale* to *Necator americanus* of greater than 20:1 based on the recovery of larval and adult hookworms from infected patients (Yong et al. 1999). Sera were obtained from Anhui residents who had high titers of circulating antibodies to *A. caninurn* L3 whole lysate antigens, as described elsewhere (Xue et al., 2000). Two of the residents were hookworm egg-negative, whereas the remaining 3 harbored quantitative fecal egg counts of less than 400 eggs per gram of feces. Because of their high antibody titer and low intensity of infection, these individuals were considered putatively resistant, and their sera were pooled and used for immunoscreening. Negative control sera were collected from college students in Shanghai.

Expression library screening: An *A. caninum* (Baltimore strain) L3 cDNA library constructed in X ZapII (Stratagene, La Jolla, CA) (Hawdon et al. 1995) was screened using the pooled antisera according to the manufacturer's instructions. Briefly, 5 x 10^4 plaques were induced to express protein by applying a nitrocellulose membrane soaked in 10 mM IPTG for 4 hr at 37 C. Following incubation, the membrane was incubated in 5% non-fat dry milk in PBS for 1 hr. The blocked membrane was incubated with a 1:100 dilution of pooled human sera in PBS for 1 hr at 22 C, washed 3 times in PBS for 10 min at 22 C, and incubated with a 1:1000 dilution of horseradish peroxidase conjugated anti-human IgG (Sigma, St. Louis MO). The membrane was developed with substrate of 3,3'-diaminobenzidine (DAB) and 0.0 15 % hydrogen peroxide. Positive plaques were subjected to several rounds of plaque purification by re-plating and re-screening. Plasmids were rescued by in vivo excision (Short and Sorge, 1992) and both strands sequenced using primers complementary to flanking vector sequence. Nucleotide and deduced amino acid sequences were compared to existing sequences in the GeneBank database by BLAST searching (Altschul et al., 1997).

Cloning of full-length Ac-MTP cDNA: All of the positive clones isolated were truncated at the 5' end. To obtain the 5' end, a PCR using a gene specific primer P I and a primer corresponding to the conserved nematode spliced leader was used to amplify the 5' end from first strand cDNA of *A. caninum* L3.Twenty μL reactions containing 100 ng of each primer, 1 U of *Taq* polymerase (Promega, Madison WI), and 1μL of cDNA was denatured for 2 min at 95 C,

followed by 30 cycles of 1 min at 94 C, 1 min at 55 C, and 2 min at 72 C. Amplicons were gel purified and cloned into pGEM Easy-T vector (Promega, Madison, WI) by standard methods.

Stage Specificity: The stage-specificity of *mtp*-1 transcription was determined by RT-PCR (Hawdon et al, 1995). *A. caninum* eggs were isolated from the feces of infected dogs by sucrose floatation (Nolan et al., 1994), axenized by treatment with NaOCl, and plated on nematode growth medium agar plates (Sulston et al. 1988). Following incubation at 26 C for 24-30 h, the hatchlings (mixed L_1/L_2) were washed from the plates with BU buffer (Hawdon and Schad, 1991) and snap-frozen in a dry ice/ethanol bath. Unhatched eggs were also snap frozen to make cDNA. *A. caninum* adults were collected from the small intestine of an infected dog at necropsy. RT-PCR was performed on *A. caninum* eggs, mixed L_1/L_2 serum-stimulated and non-stimulated L_3 (see below), and adult *A. caninum* samples as follows. Samples were ground to a powder in a pre-chilled (liquid N_2) mortar, and total RNA isolated using the TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. The RNA was treated with 10 U DNAse 1 (RNase free, Boehringer Mannheim, Indianapolis, IN) and re-extracted with TRIzol. Total egg RNA was isolated by mechanical disruption with glass beads in the presence of TRIzol using a BeadBeater machine (BioSpec, Bartlesville, OK), DNAse treated, and re-extracted as above. First strand cDNA was synthesized from each sample in a 50 μL reaction containing 50 mM Tris HCl, pH 8.3, 75 mM KCl, 3mM $MgCl_2$, 10 mM DTT, 500 ng oligo(dT) primer, 1 μg of total RNA, and 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies) at 37 C for 1 hr. The reaction was incubated at 94 °C for 5 min, and brought to 100 μL with dH_20. One μL of the first strand cDNA was used in a PCR with primers MTP5'- I (5'-CTTCTCATGATCAACAAACACTACG) SEQ ID NO: 65 and MTP3'-1 (5'AATCTAACTCCAACATCTTCTGGTG) SEQ ID NO: 66. The reaction was cycled 30 times for 1 min at 94 C, I min at 55 C, and 1 min at 72 C. Amplicons were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide.

Expression and Purification of Recombinant Protein: The full-length *Ac-mtp-1* cDNA was cloned in-frame in the expression vector pET28 (Novagen) and transformed into competent BL-21 *E. coli* cells using standard techniques. Expression of the recombinant protein, containing 6 vector-encoded histidine residues (His-Tag) at the 5' end, was induced by the addition of 1 mM IPTG for 3 hours at 37 ° C. One ml of cells expressing rMTP- 1 were sedimented by centrifugation at 5000 x g for 5 min, the supernatant discarded, and the cells

lysed in 100 mls of TE (pH 8.0) containing 100 μg/ml lysozyme and 0.1 % Triton X- 100. After incubation at 30 °C for 20 min, the sample was sonicated (power level 2-3, 20-30% duty cycle) on ice for 10 bursts of 5 sec each until the sample was no longer viscous. Soluble and insoluble cell fractions were separated by electrophoresis in a 12% SDS-PAGE under reducing conditions, and the resolved proteins visualized with Cooomassie blue staining. For purification of rMTP-1, a cell pellet from 2 l of induced bacterial culture was suspended in 60 ml of 1.0% SDS, 0.5% 2-mercaptoethanol, boiled for 5 min, and cooled to room temperature. The extract was dialyzed against 2 liters of 0.1 %SDS in PBS for 48 hr with 2 changes of buffer, and applied to a 10 ml HisBind nickel resin column (Novagen). Chromatography was conducted according to the manufacturer's instruction except that 0.1% SDS was added to all buffers.

In an effort to increase solubility and investigate the domain structure of MTP-1, 3 constructs lacking the amino HisTag sequences were made by PCR. The full length Ac-MTP cDNA (1-1642 bp), the cDNA without the 5'-propeptide (408-1642 bp), and the putative catalytic domain (408-1101 bp) were cloned in frame into pET28 at the upstream Nco I site, thereby removing the HisTag coding sequence from the vector. The recombinant proteins were expressed under the same conditions as described above. Antiserum Production Anti-rMTP polyclonal antiserum. was obtained by immunizing BABL/C mice with purified rMTP. Twenty μg of column purified rMTP was co-precipitated with alum (Ghosh et al. 1996) and injected subcutaneously. Additional boosts with alum precipitated rMTP (20 μg each) were administered at 3, 6, and 9 weeks.

Mouse antiserum was adsorbed against bacterial lysates of *E. coli* strain BL21 to remove antibodies reacting with bacterial proteins. Twenty-five ml of induced cells were centrifuged, dissolved in 25 mls of 2X sample buffer (100 mM Tris, pH6.8, 2% SDS, 2.5% 2-mercaptoethanol), and centrifuged at 12,000 x g for 10 min. Nitrocellulose membranes (4 cm x 8 cm) were soaked in the supernatant for 20 min, followed by incubation in transfer buffer (48 mM Tris, 39 mM glyine, 0.037% SDS, 20% methanol) for 30 min. The membranes were washed 3 times in PBS containing 0.1% Tween-20 and incubated with a 1:100 dilution of the mouse antiserum for 1 hr at 22 C. The incubation was repeated 2 times with fresh membranes. To confirm specificity of the antibody, an aliquot of the adsorbed mouse antiserum was adsorbed a second time against bacterial lysates of BL21 (DE3) cells expressing full length rMTP-1. The adsorbed antiserum was used for Western blotting.

In vitro activation of L3 and collection of ES products: *A. caninum* L_3 were activated under host-like conditions as described previously (Hawdon et al, 1999). Briefly, L3 collected from coprocultures were decontaminated with 1% HCllin BU buffer (Hawdon and Schad, 1991) for 30 min at 22 C. Approximately 5000 L_3 were incubated at 37 C, 5% CO_2 for 24 hr in 0.5 ml RPM_{1640}, tissue culture medium supplemented with 25 mM HEPES pH 7.0, and antibiotics (Hawdon et al., 1999) in individual wells of 24-well tissue culture plates. L3 were activated to resume feeding by including 15% (v/v) of a <10 kD ultrafiltrate of canine serum and 25 mM S-methyl-glutathione (Hawdon et al, 1995). Non-activated L_3 were incubated in RPMI without the stimuli. The percentage of feeding larvae was determined as described (Hawdon et al, 1996).

Medium containing activated and non-activated L_3 were transferred to separate microcentrifuge tubes and centrifuged for 5 min at 14,000 rpm. Supernatants from identical treatment groups were pooled, filtered through a 0.45 μm syringe filter to remove any L_3 and cast cuticles, and stored at -20 C. Prior to electrophoresis, the supernatants were concentrated by ultrafiltration using Centricon 10 cartridges (Amicon, Beverley, MA). Concentrated ES were washed with 1 ml of BU, ultrafiltered, and lyophilized.

To collect adult ES, 1260 adult worms were incubated in $RPMI_{1640}$, tissue culture medium (Hawdon et al., 1999) for 15 hrs at 37 C, 10% CO_2. The supernatant was concentrated 3-fold by ultrafiltration in Centricon 3 spin columns.

Western blotting: Lysates of bacterial cells expressing rMTP- 1 fusion proteins and lyophilized ES products were re-suspended in 2x SDS-PAGE sample buffer (4%SDS, 5% 2-mercaptoethanol, 15% glycerol) and separated on a 4-20% gradient SDS-PAG (Invitrogen, Carlsbad, CA). Separated proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA) by electroblotting at 25V for 1 hr (Towbin et al., 1979). The membrane was blocked with 5% non-fat dry milk in wash buffer (PBS, pH7.4, 0.1% Tween 20) for 1 hour at 22 C. The blocked membrane was incubated for 1 hr at 22 C with a 1:5000 dilution of mouse rMTP antisermn which has been preabsorbed against bacterial lysates expressing full length rMTP. The membrane was washed 3 times with wash buffer for 10 min at 24 °C, followed by incubation with a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-mouse Ig (Boehringer Mannheim, Indianapolis, IN) for 1 hour at 22 °C. Bands were

visualized using chemiluminescent detecting reagents (ECL+, Amersham. Pharmacia Biotech, Piscataway, NJ).

Results for Example 3A.

Cloning of *A. caninum* MTP cDNA An *A. caninum* L3 cDNA expression library was screened using pooled sera with high anti-hookworm L3 titer collected from human patients in endemic regions of China. Twelve positive clones were identified, 6 of which were identical as determined by DNA sequencing. Each clone contained a 3'poly-A tail, but was truncated at the 5' end. The 5' end was isolated from *A. caninum* L_3 cDNA by PCR using a primer derived from the nematode spliced leader (Hawdon et al., 1995; Bektech et al., 1988) together with the gene-specific primers P1.

The full length cDNA, without the poly(dA) tail, is 1703 bp (see Figure 8A, SEQ ID NO: 15) and encodes a 547 amino acid open reading frame (see Figure 8B, SEQ ID NO: 16) with a calculated molecular weight of 61,730 and a pI of 8.72. The ATG start codon begins 2 nt downstream from the end of the spliced leader sequence, resulting in a total of 23 untranslated nt at the 5' end of the *Ac-mtp-1* cDNA. A TAA stop codon is located at nt 1666-1668, followed by a 35 bp 3' UTR containing an AATAAA polyadenylation signal (Blumenthal and Steward, 1997) 12 bp upstream (bases 1687-1692) from the poly(dA) tail. Amino acids 1 through 16 of the deduced protein sequence are predicted to represent a hydrophobic signal peptide, with a potential cleavage site between Ala,6and Gly,7 (Nielson et al, 1995). The deduced sequence contains 2 potential N-linked glycosylation sites (N-X-S/T) at Asn39 and Asn159.

A BLAST search (Altschul et al., 1997)of GenBank using the Ac-MTP-1 predicted amino acid sequence indicated significant homology to members of a family of zinc metalloproteinases called the astacins (Bond and Benyon, 1995), named for the digestive protease astacin from the crayfish *Astacus astacus*. A search of the protein structure databases (Apweiler et al, 2000) with the Ac-MTP-1 deduced amino acid sequence revealed the presence of characteristic astacin fingerprints, including the extended zinc binding domain and a conserved Met turn located 37 amino acids downstream. The catalytic domain containing the zinc binding site is followed by a domain with homology to epidermal growth factor (EGF), from amino acids 334 to 368. From amino acids 374 to 484 is a domain with weak homology to the CUB domain, named for its occurrence in complement subcomponents C1r/C1s, embryonic sea urchin protein *U*egf, and *B*MP- 1. The EGF and CUB domains are common in astacin

metalloproteinases, and are believed to be involved in protein-protein interactions (Bond and Benyon, 1995).

Following the N-terminal signal peptide is a 119 amino acid, helix-rich pro-peptide domain. The C-terminal end of the propeptide domain contains a 4 basic amino acid sequence (R-E-K-R) from amino acids 132 to 135 that is a potential recognition site for furin or other trypsin-like processing enzymes (Bond and Benyon, 1995). Proteolysis at this site would activate Ac-MTP- I to a putative 412 amino acid processed form with a calculated MW of 46419 and a pI of 8.04.

RT-PCR analysis of stage specificity: The stage-specificity of *Ac-mtp-1* expression was investigated by qualitative RT-PCR of cDNA from several developmental stages of *A. caninum*. *Ac-mtp-1* specific primers were designed to amplify a 434 bp portion of the *Ac-mtp-1* cDNA corresponding to nt 985 -1419 of the complete sequence. The product of the predicted size was amplified from both non-activated and activated L_3 cDNA, but not from *A. caninum* egg or L_1/L_2 mixed stage cDNA. A band of lesser intensity was seen in adult cDNA. A longer fragment was amplified from genomic DNA, indicating that the primers spanned an intron, and confirming that the amplicons from the cDNAs were derived from amplification of cDNA rather than contaminating genomic DNA. Control primers that amplify a portion of the constitutively expressed *A. caninum* protein kinase A catalytic subunit (Hawdon et al., 1995) successfully amplified product from all DNA samples, indicating that amplifiable template was present.

Expression of recombinant MTP and immunoblotting: Recombinant MTP- 1 was produced in *E. coli*, purified by Ni column chromatography, and used to immunize BALB/c mice for the production of specific antiserum. The antiserum was adsorbed against *E. coli* lysates and used to determine if Ac-MTP-1 is secreted by *A. caninum* L_3 in vitro. ES products from 10,000 non-activated (non-feeding) and activated (feeding) L_3 were analyzed by Western blotting using the rMTP-1 antiserum. The antiserum recognizes both the full length and processed (i.e. without the pro-peptide domain) forms of rMTP-1 expressed in *E. coli* BL21 (DE3) cells but fails to recognize any bands in lysates of induced cells containing the vector alone.

The rMTP antiserum recognized bands of MW, of 47.5 and 44.5 in the ES products of 10,000 *A. caninum* L3 that had been activated to resume feeding in vitro. The antiserum failed to recognize any bands in ES from 10,000 non-activated L_3 in culture medium alone, or in adult

A. caninum ES products or worm lysates (not shown). A slower migrating band in activated ES has a MW similar to that of the processed form of rMTP (47.5 versus 46.5), indicating that *A. caninum* L_3 release processed MTP-1 during in vitro activation. The lower MW band was also recognized by pre-immune mouse serum (not shown), suggesting that the antiserum recognized a protein unrelated to Ac-MTP-1. To confirm that this recognition was non-specific, the mouse antiserum was adsorbed against BL21 (DE3) cells expressing full length MTP-1 and used to probe the Western blot. Adsorbed antiserum failed to recognize any rMTP-1, but recognized a band of MWr = 44.5 in activated ES products, suggesting that recognition of the lower MW band by the antiseruin is non-specific.

Recombinant MTP-1 was recognized by the pooled sera used to screen the library, but sera from individuals living in a non-endemic area (Shanghai) failed to recognize rMTP-1 (not shown).

Example 3B. Isolation and characterization of a MTP-1 cDNA

Serum from hookworm-infected patients in China was used as a probe to carry out the isolation and characterization of a cDNA from an *A. caninum* L3 expression library that encodes a zincmetalloprotease (Ac-mtp-1) of the astacin family. An *A. caninum* (Baltimore strain) L3 cDNA expression library constructed in l ZapII (Stratagene, La Jolla, CA) (Hawdon et al., 1995) was screened according to the manufacturer's instructions using pooled antisera from patients in Anhui Province, China, where *A. duodenale* is the predominant hookworm species (Yong et al., 1999). Sera from patients with low fecal egg counts and high titers of circulating antibodies to *A. caninum* L3 whole lysate antigens, suggesting that they might be resistant to hookworm infection, were used. Six identical, truncated clones were recovered following plaque purification. The 5' end was isolated from *A. caninum* L3 cDNA by nested PCR using the nematode spliced leader sequence together with two gene-specific primers (Hawdon et al., 1995), and two independent 5' end clones were sequenced.

Results from Example 3B.

The amplified sequence is believed to represent the complete 5' end of the transcript because the predicted ATG start codon is the first methionine following the spliced leader, the first 16 deduced amino acids encode a signal peptide characteristic of secreted proteins (Nielson et al., 1997), and alignments with similar metalloproteases suggest that this is the complete amino acid sequence. The full length cDNA, without the poly(dA) tail, is 1703 bp and

encodes a 547 amino acid open reading frame with a calculated molecular weight of 61,730 and a pI of 8.72. Amino acids 1 through 16 of the deduced protein sequence are predicted to represent a hydrophobic signal peptide, with a potential cleavage site between Ala16 and Gly17 (Nielson et al., 1997). The protein sequence contains two potential N-linked glycosylation sites (NX- S/T) at Asn39 and Asn159. A BLAST search (Altschul et al., 1997) of GenBank using the Ac-MTP-1 predicted amino acid sequence indicated significant homology to members of a family of zinc metalloproteinases called the astacins (Bond and Beynon, 1995), named for a digestive protease from the crayfish *Astacus astacus*. Members of this family are characterized by a short –terminal signal peptide that targets them for secretion, followed by a pro-peptide, and a catalytic domain containing the characteristic zinc-binding region and 'Met turn'. Unlike astacin, most other members of the family contain C-terminal domains, including variable numbers of EGF and CUB domains (Bond and Beynon, 1995). A search of the protein structure databases (Apweiler et al, 2000) with the Ac- MTP-1 deduced amino acid sequence revealed the presence of characteristic astacin fingerprints, including an extended zinc binding region, and a conserved Met turn located 37 amino acids downstream. The catalytic domain containing the zinc binding site is followed by a domain with homology to epidermal growth factor (EGF), from amino acids 334 to 368. From amino acids 374 to 484 is a domain with weak homology to the CUB domain, named for its occurrence in complement subcomponents C1r/C1s, embryonic sea urchin protein Uegf, and BMP-1 (Bork and Beckman, 1993).

Astacin metalloproteinases are synthesized as inactive proenzymes. Removal of the pro-peptide by a processing enzyme activates the enzyme (Bond and Beynon, 1995). Ac-MTP-1 contains a 119 amino acid N-terminal domain with a predicted four amino acid recognition site (R_{132} E_{133} K_{134} R_{135}) for a trypsin- or furin-type processing enzyme at its C-terminus (Bond and Beynon, 1995). Proteolysis at this site would activate Ac-MTP-1 to a putative 412 amino acid processed form with a calculated MW of 46,419 and a pI of 8.04. The pro-peptide is also predicted to contain four amphipathic α-helices separated by a short linker region (amino acids 23-86) (Kelley et al., 2000).

The stage-specificity of Ac-mtp-1 expression was investigated by qualitative RT-PCR of cDNA from several developmental stages of *A. caninum*. Specific primers were designed to amplify a 434 bp portion of the Ac-mtp-1 cDNA corresponding to nucleotides 985-1419 of the complete sequence. A product of the predicted size was amplified from both non-activated and

activated L3 cDNA, but not from *A. caninum* egg or L1/L2 mixed stage cDNA, indicating that Ac-mtp-1 is expressed primarily in the L3 stage. A band of lesser intensity was seen in adult cDNA. Although this band was weak, conclusions regarding the amount of gene expression are not possible, as the RT-PCR is qualitative only. However, a Western blot of adult lysates using mouse anti-rMTP serum failed to recognize any proteins in adult ES or lysates (not shown). This suggests that expression of Ac-MTP-1 is restricted to the L3 stage, and that the message present in the adult stages is untranslated or possibly partially degraded.

Recombinant MTP-1 was produced in *Escherichia coli*, purified by Ni column chromatography, and used to immunize BALB/c mice for the production of specific antiserum. The antiserum was adsorbed against *E. coli* lysates and used to determine if Ac-MTP-1 is secreted by *A. caninum* L3 in vitro. ES products collected from 10,000 non-activated (non-feeding) and activated (feeding) L3 (Hawdon and Schad, 1993) were analyzed by Western blotting using the rMTP-1 antiserum. The antiserum recognizes both the full length and processed (i.e. without the pro-peptide domain) forms of rMTP-1 expressed in *E. coli* BL21(DE3) cells, but fails to recognize any bands in lysates of induced cells containing the vector alone. A lower MW band was observed and is similar in size to the processed rMTP (i.e. lacking the pro-sequence), suggesting that some of the rMTP expressed in *E. coli* undergoes in vitro cleavage at the C-terminal end of the pro-peptide. This is probably the result of autocatalytic cleavage, although non-specific cleavage by a bacterial protease is also a possibility. Autocatalysis might also represent the physiological activation mechanism of Ac-MTP-1 in vivo.

The rMTP antiserum recognized bands of Mr of 47.5 and 44.5 in the ES products of 10,000 *A. caninum* L3 that had been activated to resume feeding in vitro. The antiserum failed to recognize any specific bands in ES from non-activated L3, in culture medium alone, or in adult *A. caninum* ES products or worm lysates (not shown). A slower migrating band in activated ES had a Mr similar to that of the processed form of rMTP (47.5 vs. 46.5), indicating that *A. caninum* L3 release processed MTP-1 during in vitro activation. Furthermore, MTP-1 is released only in response to stimuli that activate L3 to resume feeding, and therefore, most likely functions at some stage of the infective process (Hawdon et al., 1996). The metalloproteolytic activity described previously was also released specifically during activation,

and was of similar molecular size (Hawdon et al., 1995), suggesting that Ac-MTP-1 might be responsible for at least a portion of this activity.

A lower MW band (Mr 44.5 kDa) in activated ES products was also recognized by pre-immune mouse serum (not shown), suggesting that the antiserum recognized a protein unrelated to Ac-MTP-1. To confirm that this recognition was non-specific, the mouse antiserum was adsorbed against *E. coli* cells expressing full length MTP-1 and used to probe the Western blot. Adsorbed antiserum failed to recognize any rMTP-1, but recognized a band of Mr 44.5 in activated ES products, suggesting that recognition of the lower MW band by the antiserum is non-specific. Recombinant MTP-1 was recognized by the pooled sera used to screen the library, but sera from individuals living in a non-endemic area (Shanghai) failed to recognize rMTP-1 (not shown).

While the exact function of Ac-MTP-1 is unknown, the stage specificity of expression and the specific release during activation suggest a critical role in the infective process. Thus, interruption of Ac-MTP-1 function in vivo offers a useful strategy for the development of a vaccine to control hookworm disease.

This example demonstrates that MTP-1 is an important enzyme used by the hookworm parasite for invasion, and the protein is an immunodominant antigen because it is recognized by serum from patients with low hookworm burden despite repeated exposure to hookworm. MTP is therefore an attractive candidate for a vaccine antigen.

Example 3C. Canine vaccine trials with Ac-MTP-1 antigen

To test whether Ac-MTP-1 could be an effective vaccine, two groups of five (5) purpose-bred male beagles 8 + 1 wk of age were vaccinated either with the recombinant (expressed and isolated from *Escherichia coli*) fusion protein formulated with AS02A adjuvant, or adjuvant alone. The composition of AS02A, which has been successfully used in several malaria vaccine clinical studies, is described elsewhere (Lalvani et al, 1999; Bojang et al, 2001; Kester et al, 2001). Details of the animal husbandry and housing conditions were reported previously (Hotez et al, 2002a). The recombinant fusion protein containing a polyhistidine tag was purified from washed *E. coli* inclusion bodies that were solubilized in 6 M guanidine-HCl in 10 mM Tris HCl, pH 8.0. The solubilized inclusion bodies were processed in 5-10 ml batches by gel filtration chromatography (Sephacryl S-300, 26/60 gel filtration column [Amersham Pharmacia] pre-equilibrated in a buffer containing 0.1 NaH2PO4, 10 mM Tris-HCl

and 6 M guanidine) at room temperature (flow rate of 2 ml/minute). Selected fractions containing Ac-MTP-1 (as determined by analysis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) were pooled, refolded according to the method of Singh et al (2001), and then loaded onto a 5 ml Hi-Trap IMAC column (Amersham Pharmacia) charged with $ZnCl_2$ and equilibrated in 50 mM sodium phosphate pH 7.2, 1 M urea, and 0.5 M NaCl. The column was subsequently washed with 15 column volumes of equilibration buffer, and the bound protein was eluted with 50 mM sodium phosphate pH7.2, 1 M urea, 0.5 M NaCl, and 50 mM ethylenediamine tetraacetic acid (EDTA). Eluted samples containing protein were pooled and dialyzed against 10 mM Tris-HCl pH 8.0, 5% glycerol, 1 mM dithiothreitol, and 2 mM EDTA. The purified recombinant Ac-MTP-1 did not exhibit enzymatic activity (data not shown).

The recombinant Ac-MTP-1 fusion protein was mixed with SBAS2 adjuvant and administered to each of five dogs in four intramuscular injections on days 1, 4, 43, and 50. Each dog received approximately 140 μg of recombinant fusion protein and 0.5 ml of ASO2A per dose. Five dogs were also injected intramuscularly with AS02A on the same schedule. Following immunization, blood was collected weekly by venipuncture and the serum was separated and stored frozen at –20°C. Antigen-specific canine IgG2 and IgE antibodies were measured by indirect enzyme-linked immunosorbent assay (ELISA) as described previously (Hotez et al, 2002a). Immunoblotting of secretory products from nonactivated L3 and L3 activated under host stimulatory conditions was done as described previously (Zhan et al, 2002) using pooled sera from the Ac-MTP-1-vaccinated dogs. Fourteen days following the final immunization, each dog in the study was subcutaneosly infected with 500 *A. caninum* L3. The origin of the hookworm strain used for the study is described elsewhere (Hotez et al., 2002c). Validation of the hookworm species used in the study was confirmed by a polymerase chain reaction followed by restriction fragment length polymorphism (Hawdon, 1996). Following infection, the dogs were bled weekly by venipuncture to obtain a complete blood count (CBC). Serum chemistries were also obtained at the end of the vaccination schedule and prior to necropsy. Quantitative hookworm egg counts (McMaster technique) on each dog were obtained 3 days per wk beginning on day 12 post-infection (PI). Five wk post-infection, the dogs were killed by intravenous barbituate injection, and the adult hookworms were recovered and counted from the small and large intestines at necropsy (Hotez et al., 2002c). The

statistical significance of differences between adult hookworm burdens was determined using the Anova test, as were differences in hematological parameters and in quantitative hookworm egg counts. Comparisons of hookworm burden and egg counts with antibody titers were measured using Spearman rank order (nonparametric) correlations.

SDS-PAGE analysis of the Ac-MTP-1 recombinant fusion proteins followed by Coomassie blue staining revealed that the protein migrates with an apparent MW of 61 kDa - the predicted mass of the proenzyme. Also present is a triplet of bands that migrate with a lower apparent molecular weight, which probably corresponds to the partially processed Ac-MTP-1. Following immunization, each of the vaccine-recipient dogs developed high titers of IgG2 anti-Ac-MTP-1-specific antibody ranging between 1:40,500 and 1:364,500; the anti-Ac-MTP-1-specific IgE antibody responses ranged between 1:500 and 1:1,500. Sera from the vaccinated dogs recognized a triplet of closely migrating proteins with the predicted molecular weight of the proenzyme and processed form of Ac-MTP-1 in secretory products of host-activated L3, but not in those of non-activated L3. The additional bands may also correspond to other related metalloproteases secreted by *A. caninum* L3; at least 3 closely related expressed sequence tags from *A. caninum* L3 were found in a dbEST database (ncbi.nim.nih.gov/dbEST/index.html).

Overall, there were no statistically significant differences in the number (mean + standard deviation) of adult hookworms recovered from the vaccinated dogs (154 + 34 hookworms) compared to the number of adult hookworms recovered from control dogs (143 + 30 hookworms). However, as shown in Fig. 33A there was a statistically significant reduction in the number of adult hookworms recovered from the intestines of vaccinated dogs that had high anti-*A.caninum* IgG2 antibody titers. The Spearman correlation between antibody titers and adult hookworm burden was –0.89 ($P = 0.04$). The number of hookworms recovered from the dog with the highest antibody titer (98 hookworms) was equivalent to a 50 percent reduction in worm burden compared to the number of adult hookworms recovered from the dog with the lowest antibody titer (189 hookworms). An identical relationship was noted between IgG2 antibody titers and median quantitative egg counts (Fig. 33B).

These studies suggest that Ac-MTP-1 might offer downstream promise as an anti-hookworm vaccine antigen.

EXAMPLE 4. Canine vaccine trials with Ac-TMP, Ac-AP, and Ac-APR-1 antigens

To evaluate whether antibodies directed against parasite enzymes and enzyme inhibitors have therapeutic potential for ancylostomiasis, canine vaccine trials employing recombinant fusion proteins that encode adult *A. caninum* proteases or protease inhibitors were conducted. Because small quantities of proteins are available from living hookworms, testing these molecules as vaccine candidates requires recombinant vector expression in prokaryotic or eukaryotic host systems, followed by canine immunization with the purified recombinant fusion protein.

Material and Methods for Example 4.

Study dogs and animal husbandry: Following protocol approval by The George Washington University Institutional Animal Care and Use Committee (IACUC), purpose bred, parasite naïve, male beagles 8 ± 1 week of age were purchased, identified by ear tattoo, and maintained in the AALAC (Association for Assessment and Accreditation of Laboratory Animal Care) accredited George Washington University Animal Research Facility. The dogs were housed in a room dedicated for the study, at a room temperature of 70 ± 4°F, with 10-15 air changes per hour comprised of 100 percent fresh air, and 12 hr light cycles alternating with 12 hr dark cycles. The airflow and timer functions were monitored daily. The dogs were fed on a diet of Teklad Certified Dog Chow #8727, supplemented with a canned soft diet in the event of anorexia. The drinking water was piped from a filter plant and delivered via automatc water system; water analysis was performed by the U.S. Army Corps of Engineers. Water from the facilities automatic system is cultured for bacteria and fungi annually. The pens were flushed daily and sanitized every two weeks. Dogs within a given study group were permitted to live together and socialize prior to the hookworm larval challenge, but were caged individually post-infection. All dogs were quarantined for approximately one week before beginning the vaccine trial. Prior to vaccination a complete blood count (CBC), serum chemistries, and a pre-vaccination serum sample were obtained.

Vaccine study design and sample size: The vaccine trial was designed to test three different antigens, each formulated with alum, as well as an alum adjuvant control. A total of 24 dogs were randomly assigned into four groups comprised of 6 dogs each. The canine sample size was selected on the ability to detect a 40-50 percent reduction in the numbers of adult hookworms in the small intestines of the vaccinated group relative to control dogs, at a

statistical power of 80 percent (alpha = 0.05, two-tailed). The data were derived from the mean and standard deviation of adult hookworms previously recovered from age-matched dogs infected with 400 *A. caninum* L_3 (Hotez et al, 2002).

Recombinant Antigens: Each group of 6 dogs was vaccinated with recombinant hookworm proteins expressed as fusion proteins either in *Escherichia coli* or in an insect cell line with baculovirus. Ac-AP (Cappello et al, 1995; 1996) and Ac-TMP, were expressed in *E.coli* as pET 28 (Novagen) fusion proteins containing a polyhistidine tag (Cappello et al, 1996). Ac-APR-1 (Harrop et al, 1996) was expressed in a baculovirus pBacPAK6 vector (Clontech), modified to contain a polyhistidine-encoding sequence and additional restriction enzyme sites (Brindley et al, 2001). Recombinant Ac-AP and Ac-TMP fusion proteins were then purified by nickel affinity chromatography, followed by a second step of purification. In the case of Ac-AP (Cappello et al, 1995; 1996), the recombinant protein was purified by mono-S (Amersham-Pharmacia) ion exchange chromatography, while Ac-TMP (Zhan et al, 2002) was purified by superdex 75 (Amersham-Pharmacia) gel filtration chromatography. Ac-APR-1 (Harrop et al, 1996) was purified by substrate affinity chromatography using pepstatin agarose (Brindley et al, 2001). The antigen stock protein concentration was determined by Pierce Micro BCA assay (Pierce Chemicals) or by the absorbance of the sample at 289 nm using an extinction coefficient that was calculated from the deduced amino acid composition of the fusion protein. The amount of alum adsorbed protein in each dose of antigen was measured by the Pierce Micro BCA assay using a bovine serum albumin standard. The relative purity of each of the antigens relative to contaminating *E. coli* or insect cell proteins was determined by analysis on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Adjuvant formulations: Recombinant Ac-TMP and Ac-APR fusion proteins were alum precipitated with a combination of aluminum potassium sulfate dodecahydrate and sodium bicarbonate as described previously (Ghosh et al, 1996). The method requires the precipitation of an aqueous solution of the protein with aluminum salt under alkaline conditions, followed by centrifugation and washing (Ghosh and Hotez, 1999). Using this method, recombinant Ac-AP fusion protein was not detected in the alum precipitate. Therefore, the first two doses of Ac-AP were administered without adjuvant. However, the final two doses of Ac-AP were adsorbed to an amorphous, non-crystalline calcium phosphate gel.

Canine Immunizations: A four-dose immunization schedule was selected (Table II). Each of the dogs was vaccinated by subcutaneous immunization at two sites in the shoulder, through a 22 gauge needle. The volume of the injections ranged between 0.5 and 1.0 ml. Four doses of each antigen were administered over a 38-day period. The first two injections (primary immunization) were administered on days 1 and 4, and the final two immunizations (boosts) were administered on days 34 and 38. Dogs in the control group were injected with an equivalent amount of alum.

TABLE II. Antigen quantities and adjuvants used for each canine vaccination.

	Ac-AP	**Ac-TMP**	**Ac-APR-1**	**Alum**
Dose 1 (day 1)	100μg	71μg	12.5μg	-
Adjuvant	None	Alum	Alum	Alum
Dose 1 (day 1)	100μg	71μg	12.5μg	-
Adjuvant	None	Alum	Alum	Alum
Dose 1 (day 1)	180μg	61μg	95μg	-
Adjuvant	Calcium phosphate	Alum	Alum	Alum
Dose 1 (day 1)	273μg	69μg	86μg	-
Adjuvant	Calcium phosphate	Alum	Alum	Alum

Canine antibody measurements: Blood was collected weekly by venipuncture and the serum was separated and stored frozen at –20°C. Antigen-specific canine IgG1 antibodies were measured by indirect enzyme-linked immunosorbent assay (ELISA). Other IgG subclasses were not measured due to the unavailability of suitable high-quality canine-specific reagents. The optimal concentrations of sample sera and enzyme-linked detection antibody were determined by checkerboard titrations. Optimal antigen concentrations were determined by using a saturation technique. NUNC Maxisorp F96 certified plates (Rosklide, Denmark; Batch no. 045638) were coated with 0.1 ml per well of antigen in 0.05M carbonate bicarbonate buffer

(pH 9.6). Sealed plates were incubated overnight (ON) at 4 C and then washed 3 times with PBS (pH 7.2) using a DYNEX *Opsys* plate washer (Chantilly, VA). The plates were treated for 1.5 hours with 0.25 ml per well of 0.15M PBS (pH 7.2) containing 0.5% Tween 20 (PBS-Tween 20) at room temperature (RT), decanted, and blotted on paper towels. Various serial dilutions of test sera were prepared in 0.1 ml PBS-Tween 20 and incubated ON at 4 C. After washing, 0.1ml of anti-canine IgG1 conjugated to alkaline phosphatase (Bethyl Laboratories, Montgomery, TX) at a dilution of 1:1000 were added to each well. After 1.5 hours at RT, the plates were washed 10 times with PBS-Tween 20, before 0.1 ml of 2.5 mM of para-nitro phenylphosphate (Sigma St. Louis, MO) in a solution of 10mM diethanolamine (Sigma, St. Louis, MO) and 0.5 mM magnesium chloride (Sigma, St.Louis, MO) (pH 9.5) were added to each well. The plates were incubated in the dark for 30 minutes and read at a wavelength of 405 nm on a SpectraMax 240 PC reader (Molecular Devices, Sunnyvale, CA) with SOFTmax Pro software (Molecular Devices, Sunnyvale, Ca). The mean optical density of control canine sera was used as a baseline. The last serum dilution greater than 3 times above baseline was considered the titration endpoint. The geometric mean of these endpoints was calculated for the six canines from each group.

Canine hookworm infections and parasite recovery: Fourteen days following the final immunization, each dog in the study was orally infected with 400 *A. caninum* L_3 administered in a gelatin capsule. The origin of the hookworm strain used for the study is described elsewhere (Hotez et al, 2002). Validation of the hookworm species used in the study was confirmed by a polymerase chain reaction followed by restriction fragment length polymorphism (Hawdon, 1996). Following infection, the dogs were bled weekly by venipuncture in order to obtain a complete blood count (CBC). Serum chemistries were also obtained at the end of the vaccination schedule and prior to necropsy. Quantitative hookworm egg counts (McMaster technique) on each dog were obtained three days per week beginning on day 12 post-infection. Five weeks post-infection, the dogs were euthanized by intravenous barbituate injection, and the adult hookworms were recovered and counted from the small and large intestines at necropsy (Hotez et al, 2002). The sex of each of the adult hookworms was determined by visual inspection. The necropsies were performed over a period of three days when 8 dogs per day (two dogs from each of the four groups) were euthanized. Approximately

1-2 cm of small intestine was separated and placed into formalin for future histopathological analysis.

Statistical methods: The percentage reduction or increase in adult hookworm burden in the vaccinated group was expressed relative to the control group by the following formula:

$$\frac{(\text{mean hookworms in control group} - \text{mean hookworms in vaccinated group})}{(\text{mean hookworms in control group})} \times 100$$

The statistical significance of differences in adult hookworm burdens was determined using nonparametric tests; the Kruskal-Wallis with Dunn procedures, and Mann-Whitney *U* tests. Differences between groups in hematological parameters and in quantitative hookworm egg counts were assessed by the ANOVA test. When more than two tests were computed on the same variable, the level of significance was adjusted for the number of tests. The sex differences of the adult hookworms recovered were statistically compared by the Wilcoxon-Signed Ranks test for two dependent groups. Differences were considered statistically significant if the calculated P value was equal to or less than 0.10 (two sided) or - 0.05 (one sided).

Results for Example 4.

Adult *A. caninum* antigens: Three recombinant *A. caninum* antigens were selected for canine vaccinations. Two of them, Ac-AP and Ac-TMP are protease inhibitors secreted only by adult stage hookworms. Ac-AP is a 91 amino acid factor Xa inhibitor anticoagulant (Cappello et al, 1995; 1996), and Ac-TMP is a 140 amino acid putative tissue inhibitor of metalloproteinase, and the most abundant protein secreted by *A. caninum*. The third antigen selected, was Ac-APR-1, a 422 amino acid aspartic acid cathepsin (Harrop et al, 1996). SDS-PAGE analysis of the recombinant fusion proteins followed by Coomassie blue staining was carried out. As expected, the recombinant fusion proteins Ac-APR-1 and Ac-TMP migrated on SDS-PAGE with apparent molecular weights of M_r = 45,000 and 18,000, respectively. The predicted molecular mass of Ac-AP expressed as a pET 28 fusion protein with an N-terminal poyhistidine tag is 12,191 Da (Cappello, 1996). On SDS-PAGE, the recombinant Ac-AP fusion protein was visualized as a band with a predominant Mr of 22,000 and a minor band that migrates at approximately 15,000 Da. This observation may correspond to polypeptide

oligomer formation. This was shown previously to occur during purification of the Ac-AP natural product (Cappello et al, 1995). Factor Xa inhibitory activity, DNA sequence analysis of the pET 28 plasmid encoding the recombinant Ac-AP fusion protein, and amino terminal peptide sequence analysis by Edman degradation of the 22 kDa band confirmed the identity of the gene product (data not shown).

Canine antibody responses. A canine vaccination schedule was selected that provided for a primary immunization to be administered in two subcutaneous doses over an initial 4-day period (day 1 and day 4), followed by two subsequent subcutaneous immunization boosts that were administered beginning 30 days after the primary immunizations (day 34 and day 38). Ac-TMP and Ac-APR-1 were injected as alum-precipitated proteins. In contrast, Ac-AP did not form a precipitate with alum. Therefore, for the first two doses, Ac-AP was administered subcutaneously without adjuvant. However, during the 30-day time period between the second and third immunization, a protocol that employed calcium phosphate gel was shown to effectively precipitate Ac-AP (data not shown). For that reason, calcium phosphate was selected as the adjuvant for the final two immunizing doses of Ac-AP.

Geometric mean IgG1 antibody titers to the three vaccine antigens are shown in Fig. 34A-C. Among the dogs vaccinated against Ac-APR-1 (Fig. 34A), there was a rise in antigen-specific IgG1 following the final two immunization boosts at approximately 6 weeks after the primary immunization. In contrast, anti-Ac-TMP IgG1 antibody responses were more robust (Fig. 34B), and began to increase 2 weeks following the primary immunization, prior to the third and fourth doses. Following the final two boosts there was a second increase in anti-Ac-TMP antibody titer that exceeded 1:10,000. Five of the six dogs vaccinated against Ac-AP failed to respond immunologically to the antigen. As shown in Fig. 34C, the single canine who responded to Ac-AP vaccination exhibited an antigen-specific antibody response following the final two doses.

Adult *A. caninum* hookworm recovery from the small intestine. The numbers of adult *A. caninum* hookworms recovered from the small intestines of the vaccinated dogs is shown in Table III.. Hookworm burden reductions in the vaccinated dogs relative to dogs injected with alum alone ranged between 4.5 to 18 percent. The above reduction was not sufficient to show statistical significance between groups (Kruskal-Wallis test, $P = 0.19$). However, the probability (P) of 18 percent reduction in the number of hookworms recovered from the small

intestines of the dogs vaccinated with Ac-APR-1 (the biggest reduction in one group) was less than 0.05 by the Dunn procedure, and 0.03 by Mann-Whitney U one sided test. Dogs vaccinated against Ac-TMP also exhibited a reduction in the adult hookworm burden (10.8 percent) although this was not statistically significant. The five dogs that did not exhibit an antibody response against Ac-AP, also exhibited no significant hookworm burden reduction. However, the single dog with a significant anti-Ac-AP antibody response, exhibited a 34.7 percent reduction in adult hookworm burden. As shown in Table III, data did not provide sufficient evidence for statistically significant reductions in quantitative hookworm egg counts between the vaccinated and control dogs. Similarly, vaccination did not affect the hematological parameters of the dogs, including hematocrit, hemoglobin, white blood cell count, and eosinophilia (data not shown). As expected, the challenge dose of hookworm used in this study did not produce anemia in the control alum-injected dogs (data not shown).

Adult *A. caninum* hookworm recovery from the colon.

TABLE III. Reduction of adult hookworms in the small intestines of vaccinated relative to alum-injected dogs.

Experimental group	Dogs No.	WORMS			% Decrease
		Mean	SD	Median	
Control	6	176	22	180	
Ac-AP	5	168	36	170	4.5
Ac-AP*	1	115		115	34.7
Ac-TMP	6	157	26	161	10.8
Ac-APR-1	6	144	31	138	18**

* Positive immune response

** $P < 0.05$ (Dunn procedure)

Whereas there was a reduction in the numbers of adult hookworms recovered from the small intestines of vaccinated dogs, there was a corresponding increase in the number of adult hookworms that were recovered from the colon (Table IV).

TABLE IV. Increase of adult A caninum hookworms in the colons of vaccinated dogas relative to alum-injected dogs.

Experimental group	Dogs No.	WORMS			% Increase
		Mean	SD	Median	
Control	4	6	8	4	
Ac-AP	5	17	17	14	183
Ac-AP*	1	71		71	1083
Ac-TMP	4	36	11	32	500**
Ac-APR-1	5	24	11	27	300**

* Positive immune response

** $P < 0.05$ (Dunn procedure)

The increase in the number of adult hookworms recovered from the large intestines was statistically significant (Kruskal-Wallis test, $P = 0.07$). The dogs vaccinated with either Ac-TMP (500 percent increase) or Ac-APR-1 (300 percent increase), exhibited a statistically significant increase relative to the dogs injected with alum (Dunn procedure, $P<0.05$). Dogs that were vaccinated with Ac-AP but did not exhibit an antigen-specific antibody response did not have a statistically significant increase in the number of adult hookworms recovered from the colon. However, the single dog with a significant anti-Ac-AP antibody response exhibited a 1083 percentage increase in the number of adult hookworms in its colon.

Overall, there were no statistically significant differences between the vaccinated and control dogs with respect to the total numbers of adult hookworms recovered from small and large intestines combined (data not shown). Instead, antibody responses to the recombinant

hookworm antigens resulted in significant migration of adult hookworms away from the small intestine and into the colon. The ratio of adult hookworms in the small intestine relative to the colon decreased from 43.9 in the alum-injected dogs down to ratios between 6.6 and 7.3 in the Ac-TMP and Ac-APR-1 vaccinated dogs, respectively. The single dog exhibiting an anti-Ac-AP antibody response had a small intestine to colon hookworm burden ratio of 1.6, indicating that almost one-half of this dog's hookworm burden had shifted to the colon.

Sex-dependent differences. Hookworms of either sex did not migrate away from the small intestine and into the colon in equal numbers. As shown in Fig.35, it was more common to recover female adult hookworms from the colon than males. The greater numbers of female hookworms residing in the colon was statistically significant for dogs vaccinated with Ac-APR-1 ($P = 0.04$) and Ac-AP ($P = 0.06$). Male hookworms were more likely than female hookworms to be recovered from the small intestines, although the differences were not statistically significant. Sex determinations were not made on the hookworms attached to a 1-2 cm segment of small intestine that was saved for histopathological analysis. The mean number of hookworms in this segment ranged between 5 and 6 worms. This small number of worms did not contribute significantly to the sex-dependent difference score (data not shown).

This example demonstrates that it is feasible to vaccinate mammals with recombinant fusion proteins to elicit an antigen specific response, and that the antibody response is associate either with a hookworm burden reduction in the gut or in a shift in hookworm habitat in the gut.

EXAMPLE 5. Canine Vaccine Trials of Ac-MTP-1 and Ac-TTR

Example 5A. Antibody titers and hookworm reduction.

E. coli derived antigens Ac-MTP-1 and Ac-TTR were tested in vaccine trials in dogs. Antigens were administered with adjuvant SBAS2. The vaccinated animals exhibited high levels of canine IgG2 antigen-specific antibodies, and a modest increase in antigen-specific IgE. Subsequently the dogs were challenged by subcutaneous injection of L3 hookworm larvae.

As shown in Fig. 36A and B, there was a statistically significant reduction in the number of adult hookworms recovered from the intestines of vaccinated dogs that had high anti-*A.caninum* IgG2 anti-MTP-1antibody titers. The Spearman correlation between antibody titers and adult hookworm burden was –0.89 ($P = 0.04$). The number of hookworms recovered from the dog with the highest antibody titer (98 hookworms) was equivalent to a 50 percent reduction in worm burden compared to the number of adult hookworms recovered from the dog

with the lowest antibody titer (189 hookworms). An identical relationship was noted between IgG2 antibody titers and median quantitative egg counts.

SDS-PAGE analysis of the Ac-MTP-1 recombinant fusion proteins followed by Coomassie blue staining revealed that the protein migrates with an apparent MW of 61 kDa - the predicted mass of the proenzyme. Also present is a triplet of bands that migrate with a lower apparent molecular weight, which probably corresponds to the partially processed Ac-MTP-1. Following immunization, each of the vaccine-recipient dogs developed high titers of IgG2 anti-Ac-MTP-1-specific antibody ranging between 1:40,500 and 1:364,500; the anti-Ac-MTP-1-specific IgE antibody responses ranged between 1:500 and 1:1,500. Sera from the vaccinated dogs recognized a triplet of closely migrating proteins with the predicted molecular weight of the proenzyme and processed form of Ac-MTP-1 in secretory products of host-activated L3, but not in those of non-activated L3.

With respect to the use of the TTR antigen, as can be seen in Figures 37A and B, one dog with high IgE and IgG1antibody to TTR exhibited reduced (6%) hookworm burden.

This example demonstrates that vaccination of mammals with either MTP or with TTR elicit a protective antibody response, and that with high antibody titers a reduction in worm burden is observed.

Example 5B. Protection against blood loss and decrease in hookworm size due to vaccination with hookworm antigen

Animals were also tested to ascertain whether vaccination with hookworm antigens protected against blood loss. Vaccination with Ac-TTR was shown to confer significant protection against blood loss (Fig. 38A and B).Using the Mann-Whitney test, the differences in both hemoglobin (38B) concentration ($P = 0.036$) and hematicrit (38A) concentration ($P=0.009$) between the TTR and adjuvant control groups were statistically significant.

Further, the differences in hemoglobin concentration translated to a statistically significant reduction in worm size. Data was collected using an imaging system based on scans of the worms recovered from a host. Worms were photographed with a CoolSnapPro digital camera (Media Cybernetics), and the images measured in ImagePro Software using a macro to determine worm length (in mm) compared between treatments. As shown in Fig. 39 there was a statistically significant reduction in worm size (between 1 and 2 mm) among the TTR vaccinated group relative to the adjuvant control group.

This example demonstrates that vaccination with TTR, in addition to reducing worm burden, will also reduce blood loss.

EXAMPLE 6. Chimeric hookworm antigens

The protective effect of two different hepatitis B core particles expressing a peptide epitope that corresponds to amino acids 291-303 of Na-ASP-1 (also found in Ac-ASP-1) were investigated. Previously by investigation of relative hydropathy (hydrophobicity and hydrophilicity) of the predicted amino acid sequence of Na-ASP-1 and Ac-ASP-1 it was discovered that both molecules exhibit a hydrophilic sequence that modeling predicted could represent a looped-out region of the molecule. Covalent attachment of the peptide to KLH (keyhole limpet hemocyanin) confirmed that the chimeric molecule could protect mice against challenge infections.

Two different chimeric molecules expressing ASP-1 were constructed. ICC-1546 expresses ASP-1 amino acids 291-303 as a "looped out" tethered structure, whereas ICC-1564 expresses the same peptide as an N-terminal structure. Previous studies had demonstrated that mouse anti-L3 antibody recognizes ICC-1546, but not ICC-1564.

The antigenic chimeras were administered as described above with alhydrogel as adjuvant. DSM (detergent solubilized membrane extract of adult *A. caninum*) served as a negative control. Larval challenge was carried out by subcutaneous injection of L3 stage larvae.

The results showed that vaccination of dogs with either particle produced high levels of anti-particle antibody. Most of the antibody was directed against the hepatitis core antigen constituent. However, in one dog vaccinated with ICC-1546, there was a high level of anti-ASP-1 (and anti-peptide) antibody. This dog exhibited a significant reduction in hookworm burden (Table V).

Table V. Comparison of Anti-ASP-1 antibody and hookworm burden

ICC 1546	Total Hookworms	Anti-ASP-1 IgG1	IgG2
A1	139	1:800	0
A2	181	1:800	0
A3	170	1:200	0

A4	180	1:800	0
A5	118	1:1,600	1:1,600
Average A	158		
ICC 1564	Total Hookworms	Total Hookworms	IgG2
B1	135	1:100	0
B2	143	1:100	0
B3	206	1;200	0
B4	195	1:800	0
B5	217	1:400	0
Average B	179		
Alum	Total Hookworms	Total Hookworms	Total Hookworms
D1	176	0	0
D2	150	0	0
D3	161	0	0
D4	241	0	0
D5	255	0	0
Average D	191		

This example demonstrates that high antibody titers to a specific epitope associated with ASP-1 will result in reduced worm burden.

EXAMPLE 7. Antigen expression in baculovirus/insect cells and yeast

Expression of hookworm antigens in eukaryotic expression systems, such as baculovirus/insect cells and the yeast *Pichia pastoris*, have been carried out to afford maximum opportunities for obtaining soluble and bioactive recombinant proteins.

A. Expression in *Pichia pastoris*

The antigens Na-ASP-1, Ac-TTR, Ac-API, and Ay-ASP-2 have been successfully expressed with Pichia fermentation systems. Antigens were isolated with polyhistidine tags for ease of isolation.

B. Expression in Baculovirus/Insect Cell System

Antigens Na-CTL, Ac-MEP-1, Ac-ASP-2 and Ac-MTP-1 have been successfully expressed in a baculovirus/insect cell expression system. Antigens were isolated with polyhistidine tags for ease of isolation.

EXAMPLE 8. Cloning of cDNAs of *A. ceylanicum* Orthologous Antigens Ay-ASP-1, Ay-ASP-2 and Ay-MTP

Orthologous antigens from the hamster parasite hookworm *A. ceylanicum* were successfully cloned following the construction of an *A. ceylanicum* larval cDNA library.

The *A. ceylanicum* orthologue of ASP-1 was cloned by screening the *A. ceylanicum* L3 cDNA library using a 900bp ^{32}P- labeled Ac-ASP1 cDNA fragment as a probe. Screening of approximately 500,000 clones resulted in 85 positive clones. Of these 21 clones were sequenced of which 19 encoded identical cDNAs. No other orthologues of ASP-1 were found. The clones exhibited 85% identity and 92% similarity with Na-ASP-1.

By screening approximately 100,000 plaques of the *A. ceylanicum* L3 cDNA library using a 600bp ^{32}P- labeled Ac-asp-2 cDNA fragment as a probe, 30 positive clones were obtained, of which 10 were sequenced and found to be identical to Ay-ASP-2 predicted ORFs (orthologous clones).

By screening approximately 500,000 *A. ceylanicum* L3 cDNA library using a 590bp ^{32}P-labeled Ac-MTP cDNA fragment as a probe, 700 positive clones were obtained and 8 sequenced. Seven of the 8 encoded Ay-MTP-1, while one other encoded a putative isoform (Ay-MTP-2).

This example demonstrates that there is a high degree of similarity between antigens from *A. caninum* and A. *ceylanicum* hookworm species, and the data suggests a high degree of identity (>80%) amongst most of hookworm antigens.

EXAMPLE 9. Immunolocalization

Immunolocalization of some of the major vaccine antigens was carried out in sections of adult hookworms. The immunolocalizations were determined to be as follows: Ac-103 as a hookworm surface antigen, Ac-FAR-1 and Ac-API as components of the pseudocoelomic fluid, (Ac-API is also a pharyngeal protein), Ac-CP-1 as an amphidial gland protein, Ac-TMP in the excretory glands, and ASP-3 as an amphidial and esophageal protein. In addition the total proteins of the hookworm ES products localized to amphidial and excretory glands, and to the brush border membrane of the hookworm alimentary canal.

This example demonstrates that many of the hookworm antigens are exposed either on the surface of the worm or secreted by worm and are therefore susceptible to targeting by host antibodies or host immunocompetent cells.

Example 10. Human investigations conducted in Minas Gerais State, Brazil

It has been previously reported that in China and elsewhere, human hookworm infection exhibits a unique epidemiology compared with the other soil-transmitted helminthiases (e.g., ascariasis and trichuriasis) and schistosomiasis (Gandhi et al, 2001). Whereas the prevalence and intensity of these other helminth infections peak during childhood and adolescence and subsequently decline into adulthood, the prevalence and intensity of hookworm infection increases with age. In many Chinese and Brazilian villages (and presumably elsewhere) middle aged and even elderly residents exhibit the most severe infections.

The underlying immunological mechanisms accounting for this observation has been investigated. Shown in Figs. 40 and 41, CD-4 + lymphocytes were gated from the whole blood of hookworm infected residents and stimulated with either L3 soluble hookworm antigen Fig. 40) or Pichia-expressed recombinant Na-ASP-1 (Fig. 41). Host cytokine production was measured by an intracellular cytokine staining technique. Both antigens stimulated high levels of IL-10 and IL-5, but not IL-4. IL-10 is a strong immunomodulator with downregulatory, anti-inflammatory properties, and IL-4 is associated with antibody production and TH-2 type immunity. The findings suggest that hookworm infected individuals might be anergic to hookworm and possibly other antigen stimulation.

In contrast, it was shown that individuals treated for hookworm produce IL-4. This observation indicates that removal of hookworms from the intestine helps to reconstitute a patient's immunity. This is a critical observation since it suggests that in the absence of treatment a recombinant hookworm vaccine may be unlikely to function as a therapeutic vaccine in patients who are actively infected, and that anthelminthic chemotherapeutic treatment may be necessary prior to vaccination.

Further, these observations also suggest that hookworm infection might thwart otherwise successful vaccinations against such etiological agents as HIV and malaria. In regions of Subsaharan Africa where hookworm overlaps with HIV and malaria, it may become essential to monitor a study participant's hookworm status prior to HIV or malaria vaccination, and to treat those that are found to be actively infected prior to immunization.

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Example 11. Cloning, Yeast Expression, Isolation and Vaccine testing of recombinant *Ancylostoma*-Secreted Protein (ASP)-1 and ASP-2 from *Ancylstoma ceylanicum*

An estimated 740 million people in the developing countries of the tropics and subtropics are infected with the hookworm *Necator americanus* or *Ancylostoma duodenale* [1]. The highest prevalence of hookworm in-fection occurs in impoverished rural areas of sub-Saharan Africa, Southeast Asia, China, Brazil, and Central America [1, 2]. In some of these

regions, up to 57% of the moderate and severe iron deficiency anemia (IDA) is attributable to hookworm infection [3–5], which results from parasite-induced blood loss and hemoglobin digestion [6–8]. IDA accounts for huge numbers of disability-adjusted life-years lost in developing countries; some studies rank IDA among the top 15 causes of global disease burden [9, 10]. Because it is linked to a major etiology of IDA, hookworm infection is considered, in terms of DALYs, to be one of the most important parasitic diseases of humans, possibly second only to malaria [11]. At present, the major approach to hookworm control relies on frequent and periodic dewormings through the administration of albendazole and other anthelminthic drugs. There has been significant interest by the World Health Organization (WHO) and other international organizations in conducting such interventions on a large scale [12], particularly for school-aged children, who might otherwise suffer from the physical and intellectual growth retardation effects of hookworm and other soil-transmitted helminths (STHs) [13–15].

However, unlike other STH infections (e.g., ascariasis and trichuriasis), there is an emerging body of evidence suggesting that the peak prevalence and intensity of hookworm frequently occurs among adult populations [16–18], including high rates of hookworm infection among pregnant women, in whom hook-worm- induced IDA results in adverse consequences for both the mother and the unborn fetus [19]. Therefore, the school-based anthelminthic chemotherapy programs now being pro-posed by WHO and other international health agencies to control STH infections might fail to target hookworm. Moreover, hookworm reinfection often occurs within just a few months after anthelminthic treatment [20]. This feature of human hookworm infection would also thwart the success of mass chemotherapy initiatives.

As an alternate or complementary approach to hookworm control, efforts are under way to develop a vaccine [11]. On the basis of previous success with trickle doses of third-stage infective hookworm larvae (L3) or L3 attenuated by ionizing radiation (irL3) [21], vaccine development efforts have targeted the major antigens secreted by hookworm L3 at entry into the host [22]. The most abundant antigens released by hookworm L3 by host stimulation with serum have now been cloned from the dog hookworm *A. caninum,* including a zinc metalloprotease of the astacin class [23] and 2 *Ancylostoma*-secreted proteins (ASP-1 and ASP-2) that belong to the pathogenesis-related protein (PRP) superfamily [22, 24–25]. To test whether these antigens protect laboratory animals against challenge infections, we have adopted

a parallel strategy of testing them as immunogens in dogs challenged with *A. caninum* and in hamsters challenged with *A. ceylanicum.* Both systems offer unique advantages and disadvantages [11]. Here, we report the cloning and yeast expression of *asp-1* and *asp-2* cDNAs from *A. ceylanicum,* the isolation of the recombinant macromolecules, and their vaccine testing in hamsters. We report that vaccination of hamsters with recombinant ASP-2 formulated with the ad-juvant Quil A results in protection, as evidenced by reduction in hookworm burden, hookworm size, and spleen size, compared with those in control hamsters vaccinated with Quil A alone.

MATERIALS AND METHODS for Example 11.

Cloning of *asp-1* and *asp-2* cDNAs from *A. ceylanicum*

To construct an *A. ceylaninum* L3 cDNA library, L3 of *A. cey-lanicum* were obtained from coprocultures of a donor dog infected with *A. ceylanicum.* Total RNA was exacted from *A. ceylanicum* L3 by use of the TRIzol reagent (GIBCO BRL), and mRNA was isolated by use of oligo dT affinity chromatography (Oligotex mRNA Mini Kit; Qiagen). A l ZAPII cDNA library was constructed according to the manufacturer's instructions (Stratagene). Products of the polymerase chain reaction (PCR) from *A. caninum* cDNA and *asp*-specific primers were used as heterologous probes to screen the *A. ceylanicum* library [24, 25]. The species derivation of each hookworm reagent was abbreviated as follows: *Ac, A. caninum; Ad, A. duodenale; Ay, A. ceylanicum;* and *Na, N. americanus.* Specific primers for *Ac-asp- 1* DNA *(Ac-asp-1* F1: 5 - GCTCTCCGGCTGGTGG-3 (SEQ ID NO: 78) and *Ac-asp-1* R1: 5 - TTAAGGAGCGCTGCACAAGCC-3 (SEQ ID NO: 79)) were used to amplify *Ac-asp-1* cDNA (366–1275 bp). Specific primersfor *A. caninum asp-2* DNA *(Ac-asp-2* F1: 5 - GGGAATTCA-ATTCTATGAGATGCGGAAA- 3 (SEQ ID NO: 80) and *Ac-asp-2* R1: 5 - TGTCT- AGATAGCCACGCACGACGCAAA G-3 (SEQ ID NO: 81)) were used to amplify *Ac-asp-2* cDNA (66–668 bp). The first-strand *A. caninum* L3 cDNA reverse transcribed from A. caninum L3 total RNA was used as a template. The PCR products were labeled randomly with a ^{32} [P]-dCTP by use of a Rediprime labeling kit (Amersham). The radiolabeled 909-bp *Ac-asp-1* fragment and the 602-bp *Ac-asp-2* fragment were used as probes to screen the *A. ceylanicum* L3 cDNA library. Approximately 1 x10^5 plaques of the *A. ceylanicum* L3 cDNA library were plated on 2 NZY agar plates. Plaque DNA was transferred to positively charged nylon membranes. After denaturation with alkali and stabilization by baking for 2 h at 80°C ,

the membranes were prehybridized for 2 h at 65°C and then hybridized for 16 h in a solution of Rapid-hyb buffer (Amersham). Positive plaques were rescreened once, and the single positive clones were in vivo excised to phagemids by use of a helper phage (Stratagene). Double-strand sequencing was performed on the phagemid DNA by use of the generic vector primers T3 and T7 . Sequence editing, alignments, and comparisons were performed by use of Eyeball Sequence Editor software (version 1.09e).

Subcloning into *Pichia pastoris*

cDNA fragments encoding *Ay*-ASP-1 and *Ay*-ASP-2 (except for the predicted signal sequence) were amplified from pBluescript phagemids by use of specific primers for *Ay-asp-1* (SEQ ID NO: 55*) : (Ay-asp-1)* F1: 5 -CTCTCGAGAAAAGAAGCCCAGTAAAGCCAGC-3 (SEQ ID NO: 70) and *Ay-asp-1* R1: 5 -TGTCTAGAGGAGCAC TGCACAATC-CTT C-3) (SEQ ID NO: 71) and *Ay-asp*-2 (SEQ ID NO: 57*) (Ay-asp-2* F1: 5 - GGGAATTCGGAAA-TAATGG AATGACCG-3 (SEQ ID NO: 72) and *Ay-asp-2* R1: 5 -TGTCTAGACCATGCACG-ATGCAAA GC-3) (SEQ ID NO: 73). The PCR products were then cloned into the eukaryotic expression vector pPICZαA (Invitrogen) by use of *Xho*I/*Xba*I sites for *Ay-asp-1* and *Eco*RI/*Xba*I sites for *Ay-asp-2* (SEQ ID NO: 57*)* . The correct open-reading frame (ORF) was confirmed by sequencing that used the vector flanking primers corresponding to the regions encoding the a -factor and 3' AOX1. The recombinant plasmids were linearized by use of *Sac*I digestion and were transformed into the *P. pastoris* X33 strain by eletroporation, according to the manufacturer's instructions (Invitrogen). The transformants were selected on zeocin-resistant YPDA plates and identified by PCR amplification using the *Ay-asp*-1– and *Ay-asp*-2–specific primers *(Ay-asp-1* F1/*Ay-asp-1* R1 and *Ay-asp-2* F1/*Ay-asp-2* R1, respectively).

Fermentation and Expression of *Ay*-ASP-1 (SEQ ID NO: 56) and *Ay*-ASP-2 (SEQ ID NO: 58)

A culture inoculum was prepared from *P. pastoris* cells containing either the *Ay-asp-1* or *Ay-asp-2* gene in pPICZaA (In-vitrogen). The inoculum was prepared in 2 stages. In the first stage, 50 mL of buffered-complex glycerol medium with yeast (0.1 mol/L potassium phosphate buffer [pH 6.0] containing 1% [wt/vol] yeast extract, 2% [wt/vol] peptone, 1.34% [wt/ vol] yeast nitrogen base without amino acids, 1% [vol/vol] glycerol, and 4 x 10^{-5} % d-biotin) in a 250-mL flask was inoculated with *P. pastoris* cells and grown for 24–36 h at 30°C, to a final OD600nm of 10–20. In the second stage, 100 mL of buffered-complex glycerol medium without yeast

extract was inoculated in a 500-mL shaker flask with 5–10 mL of *P. pastoris* cells from the first-stage culture and grown for .16 h at 30 °C, to a final OD600nm of 15–20. A Bioflo 3000 fermentor (New Brunswick Scientific), with a working volume of 5 L, was used for scale-up fermentation. Growth of *P. pastoris* in the fermentor was divided into glycerol and methanol phases.

Glycerol phase. Approximately 50 mL of the shaker flask culture of *P. pastoris* cells was used to inoculate 2 L of heat-sterilized basal salt media (BSM) containing 2.5 mL/L filter-sterilized trace element (PTM1) solution. Each liter of BSM contained 0.93 g of $CaSO_4$, $2H_2O$, 18.2 g of K_2SO_4, 14.9 g of $MgSO_4$, $7H_2O$, 4.13 g of KOH, 11.35 mL of 85% H_3PO_4, and 40 g of glycerol. The pH of the BSM was adjusted to 5.0 with 29% ammonium hydroxide. Dissolved oxygen was maintained above 35% throughout the fermentation. At 21–24 h into the initial glycerol phase (when a sharp increase in the percentage of dissolved oxygen was observed), 50% (vol/vol) glycerol was introduced into the cell culture media at a set flow rate of 15 g/L/h for 6 h. The pH of the cell culture media was then increased linearly from 5.0 to 6.0 by adding 29% ammonium hydroxide. The temperature was decreased linearly from 30 °C to 26 °C over a 2-h period before the completion of this phase. Antifoam 204 (Sigma) was also added.

Methanol phase. The methanol phase was initiated when the wet cell weight reached 225–250 g/L. Methanol was added at an initial flow rate of 1 mL/L/h, increasing to 9.0 mL/L/h over an 8-h period, and then subsequently maintained at a flow rate of 9.0 mL/L/h for 87 h. The wet cell weight was ~465 and 479 g/L for cells expressing *Ay*-ASP-1 (SEQ ID NO: 56) and *Ay*-ASP-2, (SEQ ID NO: 58) respectively.

Purification and Biochemical Characterization of *Ay*-ASP-1 and *Ay*-ASP-2

The cells were harvested, and supernatant was collected by centrifugation (8650 *g* for 20 min at 4C) by use of a Beckman JA-10 rotor (Beckman Instruments). The supernatant was then centrifuged a second time, to remove traces of cells and debris. Approximately 1.6 L of supernatant was filtered through a 0.8- m mol membrane (Fisher Scientific) and was concentrated to 200 mL by ultrafiltration by use of a 10,000 MWCO membrane (Pall Corporation); 2 L of binding buffer (20 mmol/L Tris-HCl, 5 mmol/L imidazole, and 0.5 mol/L NaCl [pH 7.9]) was added to the concentrated supernatant. The modified supernatant was then concentrated again to 200 mL by ultrafiltration, and the recombinant protein was isolated by immobilized metal ion affinity chromatography (IMAC) by use of a 1.25-mL pre-packed

HisBind column (Novagen). The columns were washed with 8 mL of HisBind buffer (20 mmol/L Tris-HCl [pH 7.9] containing 0.5 mol/L NaCl), and the recombinant proteins were eluted with a stepwise gradient of the HisBind buffer containing 5 mmol/L, 20 mmol/L, 60 mmol/L, and 1.0 mol/L imidazole, as recommended by the manufacturer. The column eluates were analyzed by SDS-PAGE by use of 4%–20% Tris-glycine gels (Invitrogen) and were stained with Coomassie brilliant blue R-250 (CBB). The column fractions containing the purified *Ay*-ASP- 1 (SEQ ID NO: 56) and *Ay*-ASP-2 (SEQ ID NO: 58) were identified by Western blot by use of an antihistidine tag monoclonal antibody (Novagen), goat antimouse IgG secondary antibody conjugated to horseradish peroxidase (ICN Biomedical), and the chemiluminescent de-tection system ECL+plus (Amersham Biosciences). Fractions containing purified *Ay*-ASP-1 (SEQ ID NO: 56) and *Ay*-ASP- 2 (SEQ ID NO: 58) were concentrated by use of Amicon Ultra centrifugal filter devices (Millipore Corporation) with 30,000 and 10,000 molecular-weight cutoffs, respectively, and were desalted by use of PD-10 Columns (Amersham). Protein concentrations were determined by use of the BCA assay (Pierce) and also by SDS-PAGE using known quantities of bovine serum albumin as a control.

N-terminal sequencing. SDS-PAGE analysis of *Ay*-ASP-1 (SEQ ID NO: 56) and *Ay*-ASP-2 (SEQ ID NO: 58) was performed on a 4%–20% gradient gel transferred to a polyvinyldene fluoride (immobilon-P) membrane (Millipore) at 250 mA for 1 h. The membrane was dried on filter paper for 15 min, soaked in 100% methanol, washed 5 times for 5 min/wash in MQ water (ultrapure water purified using the Milli-Q Water System; Millipore), and then stained with CBB. After drying, the visible bands were cut out, and –terminal amino acid sequences were obtained by Edman degradation, by use of a PE Biosystems 494 protein sequencer, at the Protein Chemistry Core Facility, Howard Hughes Medical Institute of Columbia University (New York, NY).

Irradiation of L3

Live *A. ceylanicum* L3 were irradiated with 40,000 rad in a Shepherd Mark IV Cesium 137 irradiator, model 25. To obtain homogeneity of irradiation exposure, a low exposure rate (but without an attenuator) and a moving turntable were used. The decay factor was considered while calculating the time of exposure. The irL3 were inspected by microscopy to ensure that they were actively motile and viable before and after irradiation.

Hamster Vaccinations, Measurement of Anti-ASP Immune Responses, and Parasite Challenge

Three-week-old male golden Syrian hamsters (*Mesocricetus auratus*) were vaccinated by intramuscular injection with 25 m g of either *Ay*-ASP-1 (SEQ ID NO: 56) or *Ay*-ASP-2 (SEQ ID NO: 58), each formulated with either Montanide ISA-720 (Seppic) or Quil A (Brenntag Biosector). To formulate each recombinant antigen with Quil A, 25 m g of the recombinant fusion protein was mixed, in a total volume of 95 m L, with 25 m g of Quil A, which was dissolved in 100 m L of PBS (pH 7.4). To formulate each recombinant antigen with Montanide ISA-720, 25 m g of the recombinant fusion protein was mixed, in a total volume of 60 m L, with 140 m L of Montanide ISA-720 and was shaken gently for 10 min at room temperature. The final volume was 200 m L for each antigen preparation per hamster. There were 10 hamsters in each group. The antigens were administered intramuscularly every 3 weeks on days 0, 21, and 42. An additional group of 10 hamsters was vaccinated by oral vaccination with 100 irL3 in 300 m L of PBS by use of the same vaccination schedule used for the other hamsters.

Eight days after the final vaccination, the hamsters were bled retro-orbitally, and their IgG antibody responses to each of the recombinant antigens were measured by ELISA, as described elsewhere [31], by use of anti–hamster IgG conjugated with horseradish peroxidase (Rockland) as a secondary antibody. ELISA plates were developed with o-phenylenediamine substrate. Serum antibody titers were determined by measuring the last dilution that resulted in 3 SD above background. On day 56 after the initial vaccination (14 days after the final vaccination), each hamster was infected orally with 100 *A. ceylanicum* L3. The larvae were introduced directly into the stomach by use of a gavage tube.

Measurement of Hookworm Burden, Hookworm Size, and Host Spleen Size

The hamsters were killed at days 19–21 after infection, and the intestines and spleens were removed. The spleens were weighed, fixed in formalin, and examined histologically. The adult hook-worms in the intestines were removed, placed in triethanolamine and formalin fixative [26], and counted. Worm lengths were determined digitally as follows: preserved worms were photographed by use of a Cool Snap Pro CCD monochrome camera (Media Cybernetics) attached to a computer running Image Pro Plus software (version 4.1.0.0; Media Cybernetics). A macro was written to automatically determine the object endpoints and draw a "backbone" on the image of the worm. This allowed us to measure worms that were coiled or curved. The

length of the digital line was determined automatically by use of the size (length) command in the software package. The software was calibrated by photographing a ruler at the same focal depth as the worms, and the lengths were expressed in centimeters. The measurements were exported to Microsoft Excel spread- sheets, and measurements derived from spurious images, such as debris or partial worms, were removed before analysis.

Statistical Methods

Hookworm burden reduction (vaccine protection) was defined as P = (AWC - AW I)/AWC, where P (protection) is expressed as percentage, AWC is the number of adult worms in the unvaccinated control hamsters (injected with adjuvant alone), and AWI is the number of adult worms in the hamsters vaccinated with recombinant antigen or irL3 [27]. The statistical significance of differences in adult hookworm burdens was determined by use of the Kruskal-Wallis and the Mann-Whitney U nonparametric tests. Mean spleen weights were compared by use of 1-way analysis of variance. After we determined that differences existed among the means, the Bonferroni post hoc test was used to determine which means differed. Mean lengths of adult worms were compared by use of the *t* test for 2 independent groups, assuming equal variances (Levene's test). Spleen weights and circulating hemoglobin were correlated by use of the Spearman's correlation. Differences were considered to be statistically significant if the calculated $P \leq .05$.

RESULTS for Example 11.

***Cloning of* Ay-asp-1 *and* Ay- asp-2.** From $1x10^5$ plaques screened for *Ay-asp-1,* 85 positive clones were obtained. A total of 21 positive clones were subjected to DNA sequencing. Of these, 19 sequences were identical, each encoding an ORF with homology to *Ac-asp-1* (SEQ ID NO: 55) (designated as *Ay-asp*-1). The *Ay-asp*-1 cDNA included 1322 bp, with a 3 poly(A) tail, but lacked a 5' initiation codon. The *Ay-asp*-1 cDNA encodes a predicted ORF of 424 aa that lacked 1 aa (Met) at the N-terminus, compared with *Ac*-ASP-1 (SEQ ID NO: 56). The predicted ORF of *Ay*-ASP-1 (SEQ ID NO: 56) has a calculated molecular weight of 45,748.46 Da and a predicted pI of 6.03. Two putative *N*-linked glycosylation sites were detected at Asn residues 58 and 120. Amino acid sequence comparisons among ASP-1 molecules from different species of hookworm larvae revealed that *Ay*-ASP-1 (SEQ ID NO: 56) exhibited 86% identity to *Ad- ASP-*1 (SEQ ID NO: 67) and 85% identity to both *Ac*-ASP-1 (SEQ ID NO: 18) and *Na*-ASP-1 (SEQ ID NO: 2) [28] (Figure 42).

From 1 x 10^3 plaques screened for *Ay-asp-2,* (SEQ ID NO: 57) 30 positive clones were obtained. A total of 10 were subjected to DNA sequencing. The sequences of these 10 clones were identical and encoded an ORF with close identity to the single-domain *Ac-asp-2* (SEQ ID NO: 57) cDNA cloned previously from *A. caninum* [25]. The *Ay*-asp-2 cDNA included 740 bp, with a 3 poly(A) tail, but lacked a 5 initiation codon. The cDNA encoded an ORF of 217 aa that lacked 2 aa at the N-terminus, on the basis of its alignment with *Ac*-ASP-2 (SEQ ID NO: 20). The first 20 aa comprised a hydrophobic signal peptide sequence without an N-terminal Met. The predicted ORF of *Ay*-ASP-2 had a calculated molecular weight of 24,006 Da and a predicted pI of 8.04. No putative *N*-linked glycosylation site was detected in the sequence. The amino acid sequence comparison among ASP-2 molecules from different species of hookworm larvae revealed that *Ay*-ASP-2 (SEQ ID NO: 58) exhibited 83% identity to both *Ac*-ASP-2 (SEQ ID NO: 20) and *Ad*-ASP-2 (SEQ ID NO: 68) and 61% identity to *Na*-ASP-2 (SEQ ID NO: 69) (Figure 43A). One additional amino acid (Pro) is inserted into residue 140 of *Ay*-ASP-2 (SEQ ID NO: 58), compared with other hookworm ASP-2 molecules. The placement of all cysteines was conserved among the ASP-1 and ASP-2 molecules. The cDNA sequence of Na-ASP-2 (SEQ ID NO: 82) is presented in Figure 43B.

Expression, purification, and biochemical characterization of recombinant Ay-ASP-1 and Ay-ASP-2. Both recombinant fusion proteins were secreted by *P. pastoris* during fermentation. The yields of *Ay*-ASP-1 (SEQ ID NO: 56) and *Ay*-ASP-2 (SEQ ID NO: 58) were 6 and 1 mg/L, respectively. In addition to the ORF, the recombinant *Ay*-ASP-1 (SEQ ID NO: 56) and *Ay*-ASP-2 (SEQ ID NO: 58) fusion proteins each contained C-terminal *myc* and histidine tags. N-terminal amino acid sequencing by Edman degradation of *Ay*-ASP-1 (SEQ ID NO: 56) identified a SPVKA sequence (data not shown), which is the predicted N-terminus following signal peptide removal. The *Ay*-ASP-2 (SEQ ID NO: 58) N-terminus comprised an EAEAEF expressed from the vector sequence flanking an *Eco*R1 site. This was also confirmed by Edman degradation (data not shown). The predicted molecular mass of the recombinant *Ay*-ASP-1 (SEQ ID NO: 56) and *Ay*-ASP-2 (SEQ ID NO: 58) fusions proteins, which contained these additional sequences, were 46,508 (428 aa) and 25,228 (225 aa) Da, respectively. SDS-PAGE analyses of the *Pichia*-expressed recombinant proteins during purification by IMAC showed that *Ay*-ASP-1 (SEQ ID NO: 56) and *Ay*-ASP-2 (SEQ ID NO: 58) migrated on SDS-PAGE with apparent molecular weights of 48 kDa and 30 kDa, respectively (not shown).

Hamster immune responses to vaccination. The prechallenge IgG antibody titers in response to 3 vaccinations with ASP-1 or ASP-2 formulated with either Quil A or Montanide ISA-720 and 2 vaccinations with irL3 are shown in figure 44. In response to the ASP vaccination series, hamsters developed high anti–ASP-1 (1: 364,500) and anti–ASP-2 IgG (1: 135,609) titers when Quil A was used as the adjuvant and high anti–ASP-1 (1: 631,333) and anti–ASP-2 IgG (1: 135,609) titers when Montanide ISA-720 was used as the adjuvant, but only modest anti-L3 (1: 4500) titers. Because of the absence of commercially available antihamster secondary antibodies, no other immunoglobulin classes or subclasses were measured.

Vaccination and challenge with A. ceylanicum L3. After oral challenge with 100 *A. ceylanicum* L3, statistically significant reductions in adult hookworm burden were noted among the hamsters vaccinated with either irL3 (58% reduction; $P < .001$) or *Ay*-ASP-2 (SEQ ID NO: 58) formulated with Quil A (32%; $P = .025$) (table VI).

Table VI. Hookworm burden reductions in hamsters after vaccination with Quil A alone (control group), recombinant *Ay (Ancylostoma ceylanicum)*–ASP-1 (SEQ ID NO: 56) *(Ancylostoma*-secreted protein–1) formulated with Quil A, *Ay*-ASP-2 (SEQ ID NO: 58) formulated with Quil A, or irradiated *A. ceylanicum* third-stage infective larvae (L3), followed by *A. ceylanicum* L3 challenge.

Groups	Adult hookworms, mean ± SD	Reduction relative to Quil A–injected hamsters, %	*P*, one-sided
Quil A alone	55.8 ± 12.1	---	---
Ay-ASP-1 formulated with Quil A	44.4 ± 20.7	21	.16
Ay-ASP-2 formulated with Quil A	37.9 ± 19.8	32	.025
Irradiated L3	23.4 ± 16.4	58	<.001

Statistically significant protection was not observed in hamsters vaccinated with *Ay*-ASP-1 (SEQ ID NO: 56) formulated with Quil A or with either ASP molecule formulated with the adjuvant Montanide ISA-720 (data not shown). In addition to reducing hookworm burden, as shown in table VII, vaccination with *Ay*-ASP- 2 (SEQ ID NO: 58) formulated with Quil A reduced the size of the hook-worms by 14%, relative to that of the hookworms recovered from hamsters vaccinated with Quil A alone (P <.001).

TableVII. Comparison of the mean lengths of hookworms recovered from hamsters after vaccination with Quil A alone (control group), recombinant *Ay (Ancylostoma ceylani-cum)*–ASP-1 (SEQ ID NO: 56) *(Ancylostoma*-secreted protein–1) formulated with Quil A, *Ay*-ASP-2 (SEQ ID NO: 58) formulated with Quil A, or irradiated *A. ceylanicum* third-stage infective larvae (L3), followed by *A. ceylanicum* L3 challenge.

Group	No. of worms	Length, mean ± SD, cm	Reduction in worm length, %	*P*
Quil A alone	464	0.50 ± 0.18	---	---
Ay-ASP-1 (SEQ ID NO: 56) formulated with Quil A	424	0.50 ± 0.17	0	.99
Ay-ASP-2 (SEQ ID NO: 58) formulated with Quil A	310	0.43 ± 0.18	14	< .001
Irradiated L3	217	0.47 ± 0.19	6	0.18

The hamsters vaccinated with either *Ay*-ASP-2 (SEQ ID NO: 58) formulated with Quil A or irL3 experienced statistically significant reductions in host spleen size, compared with hamsters vaccinated with Quil A alone (table VIII). After host blood loss in hamsters infected with heavy hookworm burdens, the spleen expanded in size because of an influx of hematopoietic cells replacing lymphoid tissue. The extramedullary hematopoiesis was characterized by a predominance of erythroblastic cells with deep blue cytoplasm and megakaryocytes (not shown). The spleens exhibited a statistically significant negative correlation (r = -0.5; P < .01) with host circulating hemoglobin levels. In contrast, there were no statistically significant differences

in splenic weights between hamsters vaccinated with both ASPs formulated with Montanide ISA-720 or with Montanide ISA-720 adjuvant alone (data not shown). As shown in table IX, hamsters vaccinated with either irL3 or ASP-1 formulated with Quil A also experienced less loss of body weight than did hamsters vaccinated with Quil A alone.

Table VIII. Weights of spleens recovered from hamsters after vaccination with Quil A alone (control group), recombinant *Ay (Ancylostoma ceylanicum)*–ASP-1 (SEQ ID NO: 56) *(Ancylostoma-secreted protein–1)* formulated with Quil A, *Ay*-ASP-2 (SEQ ID NO: 58) formulated with Quil A, or irradiated *A. ceylanicum* third-stage infective larvae (L3), followed by *A. ceylanicum* L3 challenge.

Group	Spleen weight, mean ± SD, g	*P*
Quil A alone	0.61 ± 0.07	---
Ay-ASP-1 (SEQ ID NO: 56) formulated with Quil A	0.52 ± 0.09	.36
Ay-ASP-2 (SEQ ID NO: 58) formulated with Quil A	0.46 ± 0.14	.025
Irradiated L3	0.40 ± 0.09	< .001

Table IX. Body-weight reductions of hamsters vaccinated with Quil A alone (control group), recombinant *Ay* (Ancylostoma ceylanicum)–ASP-1 (SEQ ID NO: 56) *(Ancylostoma*-secreted protein–1) formulated with Quil A, *Ay*-ASP-2 (SEQ ID NO: 58) formulated with Quil A, or irradiated *A. ceylanicum* third-stage infective larvae (L3).

Group	Mean (median) ± SD, g	*P*
Quil A alone	17.8 (17.0) ± 4.4	---

Ay-ASP-1 (SEQ ID NO: 56) formulated with Quil A	17.8 (16.6) ± 4.9	.94
Ay-ASP-2 (SEQ ID NO: 58) formulated with Quil A	15.5 (14.3) ± 9.2	.27
Irradiated L3	10.8 (12.4) ± 4.9	.006

NOTE. Body weights were measured at necropsy and were compared with body weights at the time of experimental infection with L3.

DISCUSSION for Example 11.

In studies performed during the 1960s, irL3 were shown to induce high levels of protective immunity in dogs, as evidenced by reduced hookworm burden and size and diminished blood loss [29]. These observations provided the basis for a commercial dog antihookworm vaccine that was marketed in Florida in 1973 and then in the eastern United States in 1974 [30]. The irL3 vaccine was later removed from commercial production because of its high cost and the requirement that the irL3 needed to maintain viability in order to release hookworm antigens [11, 21, 30]. Because administration of living L3 is not a viable strategy for human antihookworm vaccine development, an alternative approach might be to vaccinate animals with antigens secreted by living larvae; this, in turn, relies on the identification of the major L3 antigens secreted by the parasite at host entry and on cloning of the corresponding genes to produce recombinant proteins [11]. The results presented here have demonstrated that, in hamsters, recombinant ASP- 2 derived from *A. ceylanicum* L3 elicits levels of protection comparable to levels elicited by irL3.

Both *asp-1* and *asp-2* cDNAs were expressed in methanol by *P. pastoris.* The rationale for selecting yeast as an expression vector is that previous attempts to express *asp* cDNAs in *Escherichia coli* resulted in the production of expressed recombinant proteins in inclusion bodies. The *E. coli*–expressed proteins could not be refolded or solubilized. We and others have determined that *E. coli* fails to express proteins of the PRP superfamily in soluble form [11,31], most likely because their high cysteine content causes improper protein folding secondary to aberrant disulfide bond formation [11]. For instance, ASP-1 is a 45-kDa molecule containing

20 cysteines and 10 disulfide bonds in 2 PRP domains [24], whereas ASP-2 is a 24- kDa molecule containing 10 cysteines and 5 disulfide bonds in a single PRP domain [22, 25]. Other investigators have reported similar difficulties in expressing PRP superfamily proteins in prokaryotic systems [31].

One advantage of using *P. pastoris,* as opposed to other eukaryotic expression systems, such as insect and mammalian cells, is the comparatively high yields obtained from the yeast system, which allows recombinant proteins to be expressed atrelatively low cost. It is anticipated that cost will be an important factor in the manufacture of human antihookworm vaccines targeted for the poorest of the poor in developing countries [32].

The ASPs were tested in laboratory hamsters challenged with *A. ceylanicum*. Although *A. ceylanicum* is considered to be only a minor cause of hookworm in humans, it has been adapted for use in studying the pathobiology of animal hookworm infections. Among the benefits of studying *A. ceylanicum* in hamsters is that heavy infections cause host blood loss leading to anemia [33]. This makes it possible to determine whether vaccination helps to reduce blood loss, as well as hookworm burden. Because the spleen increases in size and weight with extramedullary hematopoiesis caused by blood loss and anemia, the organ can be measured as a surrogate for measuring blood loss. However, the hamster model also suffers from some disadvantages for purposes of vaccine development. First, the hookworm is not a natural parasite of hamsters, and, second, there are very few immunological reagents to study the host immune response to either vaccination or infection. ASP-2 is the first recombinant vaccine antigen that has been shown to protect a permissive host (a host in which L3 complete their development to the adult stages) against hookworm at a level comparable to irL3. This molecule exhibits a high degree of amino acid similarity to Hc24, a protective antigen isolated from the trichostrongyle *Haemonchus contortus* [34–35], as well as a single-domain ASP protective antigen from *Ostertagia ostertagi* [36] and *Onchocerca volvulus* [37–38]. In sheep, Hc24- induced protection is dependent on antigen-specific host IgE [35]. The absence of hamster-specific immunological reagents made it impossible to measure antigen-specific IgE titers, although the antigen-specific IgG titers exceeded 1: 100,000 in the present study. In contrast, ASP-1 did not elicit comparable protection in hamsters, even though it elicited a strong immune response. The modest level of protection was disappointing, given that a fusion protein composed of a histidine tag and aa 96–428 of *A. caninum* ASP-1 was effective at blocking *A. caninum* L3

migrations in mice, when it was used as a vaccine with alum [39–41]. The basis for this difference is under investigation.

The differences in protection noted between ASP-2 formulated with Quil A and ASP-2 formulated with Montanide ISA-720 are also under study. Quil A is a derivative of saponin and was chosen because it has been used successfully as an adjuvant for recombinant schistosome proteins in mice and water buffaloes [42, 43]. Montanide ISA-720 was chosen because of its previous use as an adjuvant in experimental human malaria vaccines [44, 45]. Without the benefit of available immunological reagents, however, it will be difficult to determine the qualitative differences in the immune response profiles of these 2 adjuvant formulations in hamsters.

Because of the success of the ASP-2 homologue Hc24 in sheep and, in the present study, in hamsters, ASP-2 will be considered further for development and pilot manufacture of clinical-grade recombinant protein. Parallel studies have demonstrated that a small subset of humans living in regions of China and Brazil where hookworm is endemic acquire naturally circulating anti–ASP-2 antibodies. Early indications are that these individuals exhibit low hookworm burdens and are resistant to reinfection (date not shown). Moreover, in preliminary data from our laboratory, we have found some protection against *A. caninum* infections in dogs vaccinated with recombinant *A. caninum* ASP-2 (authors' unpublished data). Together with the results reported here, these data will be used to justify moving forward to human phase 1 clinical trials with *Na*-ASP-2 (SEQ ID NO: 69) as a lead vaccine candidate.

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Example 12. Antibodies against a secreted protein from hookworm larvae reduce the intensity of infection in humans and laboratory animals

An estimated 740 million people are infected with the hookworms *Necator americanus* or *Ancylostoma duodenale* in the tropics and subtropics [1]. New data employing disability adjusted life years (DALYs) reveals that hookworm disease outranks African trypanosomiasis, schistosomiasis, dengue, Chagas disease, and leprosy in terms of disease burden [2]. The major approach to hookworm control currently relies on periodic deworming through the administration of benzimidazole anthelmintic drugs. However, rapid re-infection after anthelmintic treatment [3] and the diminishing efficacy of benzimidazoles with repeated use [4] have made the successful development of an anti-hookworm vaccine an urgent public health need.

The development of a hookworm vaccine requires an understanding of how protective immune responses are generated, both in individuals from endemic areas and laboratory animals under experimental conditions. Human and animal studies of helminth infections have established the importance of antibody-mediated protection, especially the protective role of parasite-specific IgE 5 . For example, specific IgE against helminth antigens associates with

reduced infection intensities (quantitative egg counts) to human infections with *Schistosoma* [6,7], *Trichuris* [8] and *Ascaris* [9].

Individuals with high levels of total and parasite-specific IgE had fewer and less fecund hookworms [10,11]. In laboratory animals, IgE mediates resistance to experimental schistosome infections in baboons [12], nematode infections of sheep and cattle [13,14] and nematode parasites of rodents [15]. Although the exact mechanisms by which IgE mediates protection are not known, it is thought to target degranulation of mast cells, basophils and eosinophils against the parasite [5].

With human and animal studies having established the importance of IgE-mediated protection against helminth parasites, we sought to identify antigens that elicit a strong, but not harmful, IgE response for the development of an effective hookworm vaccine. Based on the success of vaccinating laboratory animals with irradiated hookworm larvae [16,17], we examined the antibody responses of individuals living in hookworm endemic areas against the most abundant antigens released by infective larval stages (L3) of hookworms, the Ancylostoma Secreted Proteins (ASPs). The ASPs belong to the pathogenesis related protein (PRP) superfamily [18,19], and both ASP-1 and ASP-2 have been shown to be protective in rodent models of hookworm infection [20,21].

Cross-sectional studies from *N. americanus* endemic areas in Brazil and China, showed that the presence of IgE against ASP-2 associated with reduction in the intensity of infection. Subsequently, the protective role of ASP-2 in a canine experimental model of hookworm infection was confirmed. These parallel findings in humans and canines suggest that the presence of antibodies against ASP-2 results in a marked reduction in infection intensity, thus providing the strongest support yet for the development of an effective recombinant vaccine against human hookworm infection.

RESULTS for Example 12.

Hookworm infection prevalence and intensities in Brazil and China

The prevalence (95 % Confidence interval [CI]) of *N. americanus* infection in the Brazilian sample was 62% (58, 66%; $n = 245$), with a mean (95% CI) epg of 301 (222, 350). The prevalence (95 % CI) of *N. americanus* infection in the China sample 6 was 56% (51, 60%; $n = 257$), with a mean (95 % CI) epg of 971 (639, 1304). Fig. 45 shows that the middle-age and elderly age strata have the highest prevalence and intensity of infection in both samples.

Infected people generate heterogeneous antibody responses to crude hookworm extracts

Sera from each blood sample were assayed for antibodies of each isotype to preparations of *A. caninum* crude antigen extracts, including third stage larval extract (L3E), adult extract (AE), and adult excretory/secretory (ES) products. L3 ES products were not available in sufficient quantities for serological analyses. Necator infected individuals produced all four IgG subclasses (IgG1, IgG2, IgG3, and IgG4) and IgE against *A. caninum* L3E, AE, and adult ES (data not shown). There was no association between levels of these antibodies and the age, sex, or intensity of infection in either study sample. A marked heterogeneity characterized the levels of antibody isotype produced to the crude antigen preparations among individuals of the same age, sex, and gender (not shown).

Expression of recombinant, secreted ASP-2 in insect cells

Ac-ASP-2 (SEQ ID NO: 20) was secreted a concentration of approximately 2 mg.L -1 by S*f*9 cells into culture medium. The protein was purified using nickel-NTA agarose and resolved as two closely migrating bands of 24-25 kDa (not shown). Both bands were recognized by monoclonal antibodies raised to the vector-derived, C-terminal V5 and His epitopes (not shown). The five N-terminal amino acids were sequenced from both bands and they were identical: G-M-R-N-S where G-M-R is derived from the restriction site in the cloning vector, and N-S are the first two amino acids of mature7 (processed) *Ac*-ASP-2 (SEQ ID NO: 20) . Mass spectroscopy revealed the molecular weight of the major peak to be 24,492.2 Da (Fig. 46); this is in agreement with the predicted molecular weight of the secreted fusion protein (25,439.9 Da) in the absence of glycosylation. *Ac*-ASP-2 (SEQ ID NO: 20) was predicted to contain one N-linked glycosylation site at Asn-204, and treatment of the recombinant protein with PNGaseF removed the majority of protein that resolved in the upper band (data not shown). *O*-glycosidase treatment did not have an effect on the apparent molecular weight of recombinant *Ac*-ASP- 2 (SEQ ID NO: 20). Rabbit antiserum raised to ASP-2 recognized the recombinant antigen as well as a protein of the expected size in L3 extracts from *N. americanus* (not shown), indicating that *N. americanus* L3 produce a protein with immunologic similarity to *Ac*-ASP-2.(SEQ ID NO: 20). A molecular model of *Ac*-ASP-2 (SEQ ID NO: 20) based on the known structure of a PRP family member (Ves v 5 from the yellow jacket) showed that the two sequences shared significant identity in fold, despite only 26% identity at the primary

sequence level (not shown). ASP-2 retained the general $\alpha.\beta.\alpha$ core sandwich fold displayed by PRPs 22 .

IgE against ASP-2 associates with reduced fecal egg counts in infected people

Necator-infected individuals were classified into one of five profiles based on the predominant isotype response to recombinant ASP-2: (1) no isotype, (2) IgG1 only, (3) IgG4 only, (4) IgE only, or (5) combined IgG4 and IgE. Fig. 4a graphically represents the relative proportions of each antibody isotype profile from both endemic areas. The largest group consisted of infected individuals who either failed to mount an antibody response to ASP-2 (29% in both areas) or mounted an IgG4 response (34% in China and 26% in Brazil). Individuals who mounted only an IgE response to ASP-2 constituted 18% of the Chinese sample and 19% of the Brazilian sample. The 8 combined IgG4 and IgE response was consistently the smallest group (9%). As shown in immunoblots (not shown), individuals who mounted an IgE response to ASP-2 did not mount an IgG1 response. Individuals who mounted an IgG1 response did not mount an IgE response (not shown). We did not observe an IgG2 or IgG3 response to ASP-2 in the serum of any individual.

Infected individuals who were positive for IgE against *Ac*-ASP-2 (SEQ ID NO: 20) from China (Fig. 48a) and Brazil (Fig. 48b) had marked (74% and 69%, respectively) and significantly ($P < 0.001$ for both) reduced fecal egg counts compared to infected individuals who did not produce IgE against ASP-2. It should be emphasized that the presence or absence of an IgE response to ASP-2, and not the quantitative nature of the response, was associated with a significant reduction in infection intensity.

Individuals who were positive for both IgG4 and IgE to ASP-2 also had reduced (30% for China and 25% for Brazil) but not significantly ($P = 0.123$) different fecal egg counts. Sera from patients from both endemic areas were also assayed for the antibody isotype response to ASP-1, a heterodimeric protein with a duplicated PRP domain (compared with ASP-2 which has a single PRP domain). While a vigorous and heterogenous antibody isotype response was detected for IgG1, IgG3, IgG4, and IgE to ASP-1, there was no association between these responses and infection intensity, age, gender or the antibody response to crude hookworm extracts (not shown).

Vaccination of dogs with recombinant Ac-ASP-2 (SEQ ID NO: 20)confers protection against hookworm infection

Canines immunized with recombinant *Ac*-ASP-2/AS03 produced strong IgG1 and IgG2 antibody titers to recombinant *Ac*-ASP-2 (SEQ ID NO: 20)(Fig. 49). The IgE titers to *Ac*-ASP-2 (SEQ ID NO: 20) in the test canines were one log lower than the IgG1 and IgG2 titers. Dogs immunized with AS03 adjuvant alone did not generate detectable antibody responses to *Ac*-ASP-2 (SEQ ID NO: 20) prior to larval challenge. Sera from dogs vaccinated with recombinant ASP-2 immunoprecipitated native ASP-2 from biotinylated *A. caninum* extracts (L3E) (not shown), while sera from animals immunized with adjuvant alone did not precipitate any L3E proteins.

We observed a marked (69%) and significant ($P = 0.025$) reduction in fecal egg counts in animals vaccinated with ASP-2 compared with control animals (Fig. 50a). We also observed a marked (30%) and statistically significant ($P = 0.044$) reduction in adult worms retrieved during necropsy from the colon and small intestine of animals vaccinated with ASP-2 (Fig. 50b).

Sera from dogs immunized with ASP-2 but not control sera interfered with migration (30% reduction) of *A. caninum* L3 through canine skin *in vitro* (Fig. 50c). There was a strong association ($r\ 2 = 0.86$; $P = 0.037$) between adult worm burden in the intestine and the inhibitory effect of serum from vaccinated canines on the ability of *A. caninum* L3 to penetrate canine skin *in vitro* (not shown).

DISCUSSION for Example 12.

Here we show that individuals who mount an IgE response to ASP-2 have markedly reduced intensity of hookworm infection. Vaccination of dogs with recombinant ASP-2 also resulted in protection as measured by reduced fecal egg counts and decreased worm burdens. Finally, sera from dogs vaccinated with ASP-2 reduced the ability of *A. caninum* L3 to migrate through canine skin *in vitro*. This is the first study to observe an association between an antibody response to a recombinant antigen and a reduction in intensity of both human and animal hookworm infections.

The ASPs are cysteine rich secretory proteins (CRISPs) belonging to the PRP superfamily [22]. All parasitic nematodes investigated to date, including the major species of animals [18,19,23-25] and humans [26-28], secrete PRPs. Available data suggest that the PRPs play diverse roles in nematode parasitism by binding to host cells. For example, nematode PRPs interfere with neutrophil recruitment by binding to integrins [29], combat hemostasis by binding

to platelets and inhibiting their activation [30], and elicit angiogenesis *in vitro* [27]. The observation that hookworm ASP-2 is released by L3 after their stimulation with serum suggests its importance during the early stages of host larval invasion [19,31]. Therefore, specific antibody responses against ASP-2 might interrupt the physiologic function of this nematode PRP.

ASPs are the most abundant antigens recognized in host protective fractions of secretory products from nematode parasites of sheep [14] and cattle [23]. In the former study, protection was mediated by antigen-specific IgE. We now show that IgE against hookworm ASP-2 is associated with reduced infection intensity in humans. Our findings are consistent with other studies on the role of IgE in immunity to *N. americanus* [10].

Based upon the observation that sera from canines vaccinated with ASP-2 inhibited *A. caninum* L3 entry through skin *in vitro*, we strongly suspect that antibodies may be working to reduce the number of L3 that ultimately enter the gastrointestinal tract by first targeting them during cutaneous entry. Two convergent lines of evidence further support this theory. First, *asp-2* mRNA is transcribed only by the L3 stage and ASP-2 protein is released by L3 only under host stimulatory conditions [19]. Therefore, ASP-2 functions during the larva's transition from the external environment to parasitism upon entry into the host [19,31]. In addition, natural and experimental infections with schistosomes suggests that IgE is an important component in the elimination of penetrating larvae [12].

The effects of anti-ASP-2 antibody may also extend beyond direct damage to invading larvae. Dogs vaccinated with ASP-2 had a marked reduction in adult worm fecundity, and hamsters vaccinated with the *Ancylostoma ceylanicum* orthologue of ASP-2 exhibited marked reduction in both adult worm burdens and worm size [21]. ASP-2 protein is not detected in adult parasites, however the anti-fecundity effect of vaccination with ASP-2 may be attributed to immunologic damage caused to L3 that go on to mature to adulthood. As larvae mature, sexual organogenesis occurs; if larvae are damaged or immunologically attenuated, some might be expected to reach maturity but in a compromised state, e.g., sterile or sexually immature. This is consistent with the observation that some radiation-attenuated helminth larvae develop into sterile adult worms [32]. Therefore, it is likely that the anti-fecundity effect induced by vaccination with ASP-2 is a result of both fewer worms reaching adulthood in the intestine, as well as a compromised reproductive capacity of those parasites that finally reach the gut.

The major clinical manifestations of hookworm disease are the consequences of iron deficiency, anemia and hypoalbuminemia, which develop when blood loss exceeds host iron and protein intake and reserves [33] . By these mechanisms, hookworm is increasingly recognized as a major global cause of iron-deficiency anemia, the world's most important nutritional deficiency [34] . Hookworm fecal egg counts correlate positively with host blood loss, and negatively with circulating hemoglobin concentration and iron status [35] . Therefore, the observation that anti-ASP- 2 antibodies associate with reduced fecal egg counts and worms burdens hasimportant clinical implications, and support the development of ASP-2 as a hookworm vaccine.

ASP-2 fulfils many of the criteria required for an efficacious hookworm vaccine. The optimal vaccine would have the following features: (1) it would decrease the number of L3 that reach the gastrointestinal tract; (2) it would prevent development of L3 into blood-feeding adult hookworms, and (3) it would block the survival and fecundity of adult hookworms [34,36] . Achieving all three goals will likely require a combination vaccine comprised of ASP-2 from the L3, in addition to an essential proteolytic enzyme for adult hookworm blood-feeding [37,38] . Development, manufacture, and clinical testing of such a combination vaccine are in progress [36]

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MATERIALS AND METHODS for Example 12.

Patient sample

The village of Daocong is located on the north of Hainan Island, China. Five hundred and ninety-one individuals were registered with the Daocong village administration. Three inclusion criteria were applied to the sample: (1) continuous residence in the endemic area over the last two years, (2) willing and able to comply with the study protocol Including blood and fecal samples); and (3) no prior treatment for hookworm during the previous two years as determined by survey. Three-hundred and ninety-six (67%) met all inclusion criteria. The 195 individuals not participating in the study did not differ by age ($P = 0.30$), gender ($P = 0.35$), occupation ($P = 0.43$), or area of residence within the village ($P = 0.40$). All research was performed in accordance with the ethical standards of the Yale University Human Investigations Committee (protocol 10932), the Internal Review Board (IRB) of the George Washington University Medical Center (protocol 080004), and the Institute of Parasitic Diseases through a single project assurance from the National Institutes of Health. Each house

was assigned a unique household identification number (HHID) and each resident a unique personal identity number (PID). Individuals excluded from the analysis received a fecal examination and were treated for any diagnosed helminth infection.

Five hundred and twenty one individuals were enumerated in the study area of Virgem das Gracas is located in Minas Gerais State, Brazil. All research was performed in accordance with the Ethics Committee of the Centro de Pesquisas de Rene Rachou, FIOCRUZ, Belo Horizonte, Minas Gerais, Brazil (06-2002 and 02- 2002) and the IRB of the George Washington University Medical Center (090303EX). At this time, each house was assigned a unique HHID and each resident a unique PID. The 3 inclusion criteria applied to the Hainan study sample were also applied here. Four-hundred and fifty-nine (88%) individuals met all three inclusion criteria. The 62 individuals not participating in the study did not differ by age ($P = 0.66$), gender ($P = 0.33$), occupation ($P = 0.21$), or area of residence within the village ($P = 0.22$). Each house was assigned a unique household identification number (HHID) and each resident a unique personal identity number (PID). Individuals excluded from the analysis received a fecal examination and were treated for any diagnosed helminth infection. Sera from 30 volunteers from a non-*Necator* endemic area in Minas Gerias, Brazil, who were egg-negative at the time of blood draw, were pooled and used as an "endemic negative control" on each ELISA plate. Sera from 28 volunteers from the United States were pooled and used as a "non-endemic negative control" on each ELISA plate.

Recombinant protein expression

Recombinant *Ac*-ASP-2 (SEQ ID NO: 20) was expressed in *Spodpotera frugiperda* Sf9 insect cells using the pMIB-V5/His expression system (Invitrogen, Carlsbad, CA). The entire ASP-2 open reading frame (GenBank accession number AF 089728) minus the N-terminal signal peptide (from Asn-18 to the C-terminal Gly-218) was cloned into pMIB-V5/HisA using the *Sp*hI and *Xb*aI restriction sites so that the recombinant ASP-2 was fused in-frame with the vector-derived N-terminal melittin signal peptide and C-terminal V5 and 6-His epitopes. *Sf*9 cells were grown in Excell 420 medium (JRH Bioscience, Lenexa, KS) and transfected with ASP-2 plasmid midi-prep (Qiagen, Valencia, CA) and Genejammer transfection reagent (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Transfected cells were selected using Blasticidin S (Cayla, Toulouse, France) at a final concentration of 25 $\mu g.ml^{-1}$ in 6 well plates and maintained in 10 $\mu g.ml^{-1}$ blasticidin after selection. Selected cells were

transferred successively from adherent populations to shaker flasks according to the manufacturer's instructions (Invitrogen). Stably selected cells in log phase were then used to seed a total of 4 liters of Excel 420 medium to a final cell density of 1.0×10^{6} cells per ml in a Bioflo 110 bioreactor (New Brunswick Scientific, Edison, NJ) with a 7.5 liter vessel. The cells were maintained at a temperature of 27□C and stirred at 70 rpm in the presence of 55-80% dissolved O_2. pH was not adjusted and remained between 6.1-6.4. Cells were grown until a cell density of 1.0×10^{7} cells per ml was attained. Supernatant was harvested by centrifugation at 4,000 × *g* and concentrated 10-fold by ultrafiltration using a 10 kDa cut-off ultrasette membrane (Pall Corporation) and peristaltic pump. Concentrated supernatant containing recombinant ASP-2 was buffer-exchanged into milliQ H2O followed by binding buffer (0.05M NaH_2PO_4, 0.3M NaCl, 10 mM imidazole, pH 8.0) before being applied to a nickel-NTA agarose column (Novagen, EMD Biosciences, Darmstadt, Germany) with a settled bed volume of 2.0 ml. The column was washed with 10 volumes of binding buffer followed by 5 column volumes each of 20, 40 and 60 mM imidazole in binding buffer. Proteins were eluted in 5 column volumes of 250 mM imidazole in binding buffer. Fractions were assessed for recombinant protein and resulting purity by SDS-PAGE using pre-cast 4-20% Tris-glycine gradient mini gels (Invitrogen) stained with Coomassie Brilliant Blue (CBB). Fractions containing purified protein were pooled, concentrated and buffer-exchanged into PBS, pH 7.2 at 4°C. Protein concentration was determined using a micro BCA kit (Pierce, Rockford, IL).

Molecular modeling

The predicted structure of *Ac*-ASP-2 (SEQ ID NO: 20) was determined by modeling the amino acid sequence against all coordinates in the RCSB Protein Data Bank using the first-approach mode in Swiss-Model. Pdb files generated were refined and viewed using Swiss-PdbViewer 3.7.

Biochemical analyses of recombinant Ac-ASP-2

Recombinant *Ac*-ASP-2 (SEQ ID NO: 20) (2.0 μg) was transferred to PVDF membrane, stained with CBB, destained and rinsed extensively in distilled H2O before being submitted for Edman degradation at Columbia University Protein Core Facility, NY. Molecular weight determinations and purity were determined by Matrix-Assisted Laser Desorption Ionization, Time of Flight (MALDI-TOF) spectroscopy using an AXIMA-16 CFR instrument (Kratos Analytical Inc., Chestnut Ridge, NY) by Dr Paolo Lecchi at The George Washington University

Proteomics facility. The glycosylation status of recombinant *Ac*-ASP-2 was assessed using an Enzymatic CarboRelease kit (QA-Bio, San Mateo, CA) under denaturing conditions to remove any N-linked and O-linked oligosaccharides.

Production of rabbit anti-Ac-ASP-2 serum and Western blotting

Ac-ASP-2 (SEQ ID NO: 20) was formulated with Freund's Complete Adjuvant (first immunization) and Freund's Incomplete Adjuvant (second-fourth immunizations) using standard procedures. An antiserum against formulated *Ac*-ASP-2 (SEQ ID NO: 20) was raised in a rabbit by immunizing with 150 μg of recombinant protein per dose. The rabbit was immunized four times at 3 weekly intervals. Blood was drawn before the first and one week after the final immunization and sera were recovered. Western blotting was used to determine the antigenicity of recombinant *Ac*-ASP-2 (SEQ ID NO: 20) and to identify the protein in L3 extracts of *N. americanus*. Twenty-five nanograms of recombinant protein or 1.0 μg of larval extracts were separated on a 4-20% gradient SDS polyacrylamide gel and subsequently transferred to PVDF membrane. After transfer, the membrane was blocked with 5% non-fat dry milk in TBS/0.05% Tween-20 (TBST) overnight, and then probed with a 1:20,000 dilution of rabbit serum (pre- and post-vaccination) for one hour. After three washes with TBST, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (heavy and light chains) for one hour. Bands were visualized using ECL plus enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Parasitological methods

The presence of intestinal nematode eggs was determined by saline float. In the case of a positive fecal sample, 3 subsequent fecal samples were taken over the course of 3 days. Two slides from each day's fecal sample were prepared within 24 hours of receipt using the Kato-Katz thick smear technique. Hookworm species *(A. duodenale* or *N. americanus*) were determined by morphological identification of third stage larvae reared from eggs by coproculture [39] .

Indirect ELISA.

Serum samples were obtained from whole blood collected into siliconized tubes. Serum was separated by centrifugation at 800 × *g* for 10 min; the resulting serum supernatant was transferred to sterile 1 mL tubes and stored at -80 °C. An indirect ELISA was then used to study isotype responses of participants to three crude *A. caninum* antigen preparations. Nunc

Maxisorp Surface 96 well plates (Nalge Nunc International, Rochester, NY) were coated with 0.5 μg/well of crude antigen preparation in 20 mM sodium bicarbonate/27 mM sodium carbonate, pH 9.6 and stored overnight at 4 °C. For IgG2 assays, 96-well plates were adsorbed overnight at room temperature with 100 μl/well of Poly-L-lysine at 1 $\mu g.mL^{-1}$ in 50mM sodium carbonate, pH 9.0. Plates were then washed with PBS and crude antigen added and incubated in the manner described above. Plates were washed 5 times with PBS, pH 7.2, and then blocked for 1h with PBS containing 1% fetal calf serum. Plates were washed 5 times with PBST. Serum samples were diluted 1:100 in PBST, and 100 μL/well was added in duplicate. Plates were incubated overnight at 4 °C and then washed 5 times with PBST as before. One hundred μL of the following dilutions of horseradish peroxidase–conjugated anti-human antibodies (Zymed, San Francisco,18 CA) were added to each well: 1:5,000 of IgG1; 1:1,000 of IgG2, IgG3 and IgG4; and 1:800 of IgE. The plates were incubated for 1h at RT and then washed 10 times with PBST. One hundred μL per well of *Ortho*-Phenylenediamine (OPD, Sigma-Aldrich, St Louis, MO) containing 0.03% hydrogen peroxide was then added. Plates were developed for 30 minutes in the dark. The reaction was stopped with 50 μL per well of 30% H_2SO_4 and the Optical Density (OD) measured at 492 nm on an automated ELISA reader (Molecular Devices, Sunnyvale, CA). We considered sera to be positive for an isotype response to a defined antigen when the OD reading for the isotype against *Ac*-ASP-2 was 3 SD above the combined mean OD of the USA and Brazil controls. Assays were standardized according to previously described methods [40] . Sera used in the IgE-ELISA were not de-adsorbed for other isotypes because of the observation that people who produced IgE did not produce IgG1 and vice versa.

Data entry and statistical methods for human studies

All research on human subjects was conducted by trained personnel by the standards of Good Clinical Practices. Data from case report forms were double-entered into an Excel file and then transferred to SPSS 10.00. Conflicts in double entry of data were resolved by referring to source documents. Student's *t*-test was used to determine significant differences in means for normally distributed continuous variables for two independent effects. ANOVA was used to test the mean differences of continuous variables when an effect consisted of more than one group (e.g., 10- year age intervals). Bonferroni *post hoc* tests, with a significance level of 0.05, were used for multiple pair-wise testing normally distributed continuous variables. A chi-square test was used to test the significance of proportions of egg positive and negative individuals. A

Pearson product moment correlation was used for all19 correlations among normally distributed variables. Spearman correlation coefficient was used as a nonparametric measure of correlation between ordinal variables. For all of the cases, the values of each of the variables were ranked from smallest to largest, and the Pearson correlation coefficient was computed on the ranks. Before calculating a correlation coefficient, data were screened for outliers.

Animal husbandry and vaccination

The test and control animals were purpose bred, parasite naïve male beagles 56 ± 4 days of age (body weights equal to or exceeded 2.5 kg, hematocrit equal to or exceeded 28.0, WBC did not exceed 20×10^6) on arrival. All dogs in a trial were purchased from the same vendor (Marshall Farms, North Rose, NY), identified by ear tattoo, and maintained in the George Washington University Animal Research Facility as previously described [37]. The experiments were conducted according to a protocol approved by the George Washington University Animal Care and Use Committee. Dogs were housed in groups for approximately one month and 1 to 3 animals per cage thereafter; controls were housed identically to test animals. Following larval challenge, all dogs were individually housed. A serum sample was obtained from each dog before the first and after each subsequent vaccination.

Crude hookworm antigen preparation.

A. caninum L3 were harvested and homogenized to generate soluble extracts (L3E) as previously described [41]. Adult *A. caninum* ES proteins and somatic extracts (AE) were prepared as previously described [25,42]. *N. americanus* L3 were harvested and soluble extracts prepared as described elsewhere [43]. Protein concentrations were measured using the BCA protein assay kit (Pierce).

Vaccine study design and antigen-adjuvant formulation

This study was conducted and reported in compliance with the intent of the Good Laboratory Practice Regulations (F.R. Vol. 43, No. 247, pp. 60013-60025, Dec. 22, 1978 and subsequent amendments). The study was audited by Quality Assurance while in progress to assure compliance with GLP regulations, adherence to the protocol and standard operating procedures. The data and final report were audited by Quality Assurance to assure that the report accurately described study conduct and results. The vaccine trial was designed to test *Ac*-ASP-2 formulated with Adjuvant System 03 (AS03) 44 obtained from GlaxoSmithKline . The rationale for selecting AS03 as an adjuvants is discussed elsewhere 37 . The ten purpose bred

beagles were randomized into two arms: immunized with *Ac*-ASP-2 (SEQ ID NO: 20) or adjuvant only (control). To make six doses of *Ac*-ASP- 2 (SEQ ID NO: 20) formulated with AS03, 600 μg of recombinant protein (0.3 ml of *Ac*-ASP-2 (SEQ ID NO: 20) at a concentration of 2 mg.ml -1) was mixed with 1.2 ml of 20mM Tris-HCl, 0.5 M NaCl, pH 7.9 and 1.5 ml of AS03; the contents of the tube were vortex mixed for 30 seconds then shaken at low speed for 10 minutes. Dogs were immunized with 100 μg of formulated antigen in a final volume of 0.5 ml. AS03 only control was prepared as described above, with PBS included instead of *Ac*-ASP-2 (SEQ ID NO: 20). Formulation of GSK adjuvants were conducted according to the protocol provided by GSK. All injections were performed intramuscularly (IM). Test and control articles were prepared on the day of injection.

Hookworm infections and parasite recovery

A. caninum larvae were cultured from eggs collected in the feces of infected dogs. All hookworms in the infective challenge were approximately equal age (17±7 days post hatching). The species identity of the infective larvae were validated using PCR 45 . All dogs were infected by the footpad method with the same dose of 500 L3 of *A. caninum* 37 . Larval challenge occurred on one of three consecutive days (at age 120 ± 9 days). Fourteen-sixteen days after the final immunization, dogs were anaesthetized using a combination of ketamine and xylazine (20mg.kg $^{-1}$ and 10mg.kg $^{-1}$ respectively), and 500 *A. caninum* L3 in a final volume of 50 μl were applied to the footpad.

Canine immunizations and antibody measurements

Beagles were immunized with formulated *Ac*-ASP-2 (SEQ ID NO: 20) as previously described [37] . The vaccines were administered IM three times beginning at age 62 ± 4 days. Boosts were administered to the dogs at intervals of 21 days. Blood was drawn at least once every 21 days and serum was separated from cells by centrifugation. Each animal's specific antibody response was evaluated by indirect ELISA using serum taken prior to the infective challenge [37] . Recombinant *Ac*-ASP-2 (SEQ ID NO: 20) was coated onto microtiter plates at a concentration of 5 μg.ml $^{-1}$. Dog sera were titrated between 1:100 and 1:2 × 10 6 to determine endpoint titers. Anti-canine IgG1, IgG2 and IgE antibodies conjugated to horse-radish peroxidase (Bethyl Laboratories, Montgomery, TX) were used at a dilution of 1:1,000.

L3 skin penetration assays

Live *A. caninum* L3 were incubated with sera (neat) from immunized dogs then L3 were placed on canine skin to observe whether serum antibodies interfered with the penetration of skin *in vitro* [46] . Briefly, fresh skin from the ear of a dog was shaved, and approx. 4 cm 2 section of skin was stretched and sandwiched between 2 × 20 mL syringe barrels that were clamped together with bulldog clips. The lower syringe was filled to the top with PBS so that the buffer was in contact with the underside of the skin. One milliliter of PBS was placed on the skin for 15 min to check integrity of the skin. L3 (300 L3/group) were then incubated in 0.05 ml of PBS, pH 7.2, or undiluted serum from different vaccinated or control dogs for 30 min at 37 °C. Each group of L3 were then placed on the upper side of the skin (added to the 1.0 ml of PBS already present) and allowed to migrate for 30 min at RT. L3 that remained on the surface of the skin were collected and counted, by removing the remaining liquid with a pipette and washing the skin with 2 volumes of PBS. Each assay was performed in triplicate.

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EXAMPLE 13. Vaccination of dogs with a recombinant cysteine protease from the intestine of canine hookworms diminishes fecundity and growth of worms

Hookworms digest blood-derived hemoglobin using a range of mechanistically distinct proteases, and preliminary data suggested that *Ac*-CP-2, a cathepsin B cysteine protease [6] from *A. caninum* might be involved in this pathway [15]. With a view to eventually vaccinating people against human hookworm disease, we decided to immunize dogs against the canine hookworm, *A. caninum*, with catalytically active recombinant *Ac*-CP-2 to determine whether vaccinated animals were protected against hookworm disease. We show that the cathepsin B-like protease, *Ac*-CP-2 is secreted as a proteolytically active enzyme by the yeast *Pichia pastoris* and that the enzyme is expressed in the intestinal lumen of blood-feeding adult hookworm parasites. Vaccination of dogs with *Ac*-CP-2 formulated with several discrete adjuvants resulted in reduced fecal egg counts and decreased sizes of female and male worms. Moreover, the number of female hookworms present in the intestines of vaccinated dogs was significantly reduced relative to control dogs. Antibodies generated by vaccinated dogs bound to the intestinal lumen and intestinal contents of hookworms recovered from those dogs, and interfered with proteolytic function of the recombinant *Ac*-CP-2 enzyme *in vitro*.

MATERIALS AND METHODS for EXAMPLE 13.

Expression of recombinant *Ac*-CP-2 in *Pichia pastoris*

The entire open reading frame encoding the pro-enzyme of *Ac*-CP-2 (spanning Ala-12 to the C-terminal Val-340) excluding the predicted signal peptide was cloned into the expression vector pPIC-Zα using the *Xba*I and *Xho*I sites. Colonies were selected from transformed cells and suspension cultures were grown in flasks then transferred to a Bioflo 3000 fermentor (New Brunswick Scientific) utilizing a 5 liter vessel as described [1]. The recombinant protein was secreted into culture medium and affinity purified on nickel-agarose as described [1].

Assessment of catalytic activity and glycosylation of recombinant *Ac*-CP-2

Purified, recombinant *Ac*-CP-2 was assessed for proteolytic activity using the fluorogenic peptidyl substrate Z-Phe-Arg-aminomethyl coumarin (AMC) (Bachem) [2]. The pH optimum of *Ac*-CP-2 was assessed using Z-Phe-Arg-AMC at different pH values according to published protocols [3]. The cysteine protease inhibitor E64 was included in some assays at a

final concentration of 5 μM. Recombinant *Ac*-CP-2 was treated with *P*NGase F and O-glycosidase, according to the manufacturer's instructions (Enzymatic CarboRelease kit, QA-Bio), under denaturing conditions to remove any N-linked and O-linked oligosaccharides.

Animal husbandry and vaccination

Purpose-bred, parasite naive, male beagles aged 8±1 wk were purchased from Marshall farms, identified by ear tattoo, and maintained in the George Washington University Animal Research Facility as previously described [4]. The experiments were conducted according to a protocol approved by the University Animal Care and Use Committee. Before the first vaccination and after each subsequent one, a serum sample was obtained from each dog.

Vaccine study design and antigen-adjuvant formulation

The vaccine trial was designed to test *Ac*-CP-2 formulated with 4 different adjuvants. ASO3 and ASO2 were obtained from GlaxoSmithKline and ISA 70 was obtained from SEPPIC, Inc. Alum was prepared as described [5]. To make six doses of *Ac*-CP-2 formulated with ASO3, 600 μg of recombinant protein (0.3 ml of *Ac*-CP-2 at a concentration of 2 $mg.ml^{-1}$) was mixed with 1.2 ml of 20mM Tris-HCl, 0.5 M NaCl, pH 7.9 and 1.5 ml of ASO3; the contents of the tube were vortex mixed for 30 seconds then shaken at low speed for 10 minutes. Dogs were immunized with 100 μg of formulated antigen in a final volume of 0.5 ml. To make six doses of *Ac*-CP-2 formulated with ASO2, 600 μg of recombinant protein (0.3 ml of *Ac*-CP-2 at a concentration of 2 $mg.ml^{-1}$) was mixed with 0.9 ml of 20mM Tris-HCl, 0.5 M NaCl, pH 7.9 and 1.8 ml of ASO2; the contents of the tube were vortex mixed for 30 seconds then shaken at low speed for 10 minutes. Dogs were immunized with 100 μg of formulated antigen in a final volume of 0.5 ml. To make six doses of *Ac*-CP-2 formulated with ISA 70, 600 μg of recombinant protein (0.3 ml of *Ac*-CP-2 at a concentration of 2 $mg.ml^{-1}$) was mixed with 1.66 ml of ISA 70; the contents of the tube were vortex mixed for 30 seconds then shaken at low speed for 10 minutes. Dogs were immunized with 100 μg of formulated antigen in a final volume of 0.327 ml. To make six doses of *Ac*-CP-2 formulated with alum, 600 μg of recombinant protein (0.3 ml of *Ac*-CP-2 at a concentration of 2 $mg.ml^{-1}$) was mixed with 0.135 ml of 1M NaHCO3; 0.3 ml of $AlK(SO_4)_2\ 12H_2O$ [5] was added to initiate precipitation. Precipitate was collected by centrifugation at 14,000 rpm for 10 mins. The supernatant was collected and the precipitation was repeated; the supernatant was collected and assayed for non-precipitated protein using a BCA protein assay (Pearce). The two precipitates were pooled,

washed with PBS and resuspended in 3 ml of the supernatant and dogs were immunized with 100 μg of formulated antigen in a final volume of 0.5 ml. Alum only control was prepared as described above, with PBS included instead of *Ac*-CP-2.

Canine immunizations and antibody measurements

Beagles were immunized with formulated *Ac*-CP-2 as previously described [4]. The study regimen used is shown along the X-axis of Figure 2. The vaccines were administered intramuscularly three times beginning at age 62 +/- 4 days. Boosts were administered to the dogs at intervals of 21 days. Blood was drawn at least once every 21 days and serum was separated from cells by centrifugation. Enzyme-linked immunosorbent assays (ELISA) were performed as previously described [4]. Recombinant *Ac*-CP-2 was coated onto microtiter plates at a concentration of $\mu g.ml^{-1}$. Dog sera were titrated between 1:100 and 1:2×10 6 to determine endpoint titers. Anti-canine IgG1, IgG2 and IgE antibodies conjugated to horse-radish peroxidase (Bethyl Laboratories) were used at a dilution of 1:1,000.

Hookworm infections and parasite recovery

Fourteen-sixteen days after the final immunization, dogs were anaesthetized using a combination of ketamine and xylazine (20mg/kg and 10mg/kg respectively) and 500 *A. caninum* L3 in a final volume of 50 μl were applied to the footpad. After applying L3, the foot was wrapped in parafilm, gauze padding and packaging tape in that order to ensure that L3 did not escape from the site of application. Dogs were monitored for 3 hours after which the parafilm, gauze and tape were removed. The site of L3 application was rinsed with saline and any remaining L3 that had not penetrated were counted. Quantitative hookworm egg counts (McMaster technique) were obtained for each dog 3 days per week beginning on day 13-15 post-infection. Four weeks post-infection, the dogs were killed by intravenous injection of barbiturate, and adult hookworms were recovered and counted from the small and large intestines at necropsy [4]. The sex of each adult worm was determined and worm lengths were measured as described elsewhere [61Approximately 1–2 cm lengths of the small intestine were removed and stored in formalin for future histopathological analysis.

Statistical methods

The percentage reduction or increase in adult hookworm burden in the vaccinated groups was expressed relative to the control group as described elsewhere [4]. The statistical significance of differences in adult hookworm burdens was determined using nonparametric tests: the

Kruskal–Wallis test with Dunn procedures,and Mann–Whitney U-tests. Differences between groups in quantitative hookworm egg counts and worm lengths were assessed by the ANOVA test. Once determined the differences among the means of groups were determined, a Dunnet post hoc multiple comparison t test was used to compare the vaccine treatment groups against the control group. The sex differences of the adult hookworms recovered were statistically compared using the Wilcoxon–Signed Ranks test for 2 dependent groups. Differences were considered statistically significant if the calculated *P* value was equal to or less than 0.10 (2-sided) or -0.05 (1-sided).

Immunohistochemistry

Adult hookworms recovered from vaccinated dogs were fixed, sectioned and probed with various sera and Cy3-conjugated secondary antibodies (BD Biosciences) as previously described [7]. Sera from vaccinated dogs and Cy3-conjugated anti-dog IgG were diluted 1:500. Some sections were probed with rabbit anti-*Ac*-CP-1 serum [6] followed by Cy3-conjugated anti-rabbit IgG; both antibodies were diluted 1:500.

Effect of anti-*Ac*-CP-2 IgG on proteolytic activity

Canine IgG was purified from sera of vaccinated dogs using protein A-agarose (Amersham Biosciences) as previously described [8]. Purified IgG (10–500 ng) was incubated with 1.0 μg of recombinant *Ac*-CP-2 for 45 mins prior to assessing proteolytic activity as described above.

RESULTS for EXAMPLE 13.

Secretion of catalytically active, glycosylated *Ac*-CP-2 by *P. pastoris*

Ac-cp-2 cDNA (GenBank accession number U18912) was cloned and reported by Harrop and colleagues [6]. We expressed recombinant *Ac*-CP-2 as a secreted fusion protein in *P. pastoris* with a yield of 35 mg.L $^{-1}$. Secretion was mediated by the α-mating factor signal peptide derived from the pPIC-Z α vector. The protein was purified from *P. pastoris* culture supernatant using nickel-agarose [20]. The purified protein migrated with an apparent molecular size of 48 kDa (not shown). This was higher than the predicted size of the pro-enzyme (41.8 kDa) and processed, mature enzyme (32.1 kDa) factoring in the C-terminal *myc* and His tags and –terminal EAEAEF motifs (introduced by the choice of restriction sites used in cloning of the construct). N-linked glycosylation of the 5 predicted sites in *Ac*-CP-2 probably accounted for some of the discrepancy between the predicted and observed molecular sizes. Deglycosylation with P*N*GaseF reduced the apparent molecular mass of recombinant *Ac*-CP-2

by 5-10 kDa although numerous bands within this size range were apparent (not shown), probably corresponding to partially deglycosylated proteins. N-terminal amino acid sequencing of the major secreted protein by Edman degradation showed the N-terminal residue to be Glu-13, suggesting that some post-translational processing of the pro-region had occurred. However, this did not correspond with the predicted cleavage site of the pro-region from the mature enzyme (Asp-81 - Asp-82 using the numbering scheme of the fusion protein presented here). Although this is only a predicted cleavage site based on the known cleavage site of the pro-region of other related enzymes [9], it is unlikely that Glu-13 is the N-terminal residue of the native, secreted protease. Difficulty in obtaining sufficient quantities of native, hookworm-derived *Ac*-CP-2 precluded N-terminal sequence information for comparison. Nonetheless, numerous faint bands with molecular sizes ranging from 30-40 kDa appeared when the purified recombinant *Ac*-CP-2 was stained with silver (not shown), suggesting that a small quantity of the recombinant protein was correctly processed to yield the mature form of the enzyme. This was further confirmed by the catalytic activity seen when recombinant *Ac*-CP-2 was incubated with Z-Phe-Arg-AMC (Figure 51). A broad pH range was observed with activity detected between pH 4-8 with optimal catalysis between pH 5 and pH 7. Addition of the cysteine protease inhibitor, E64, to a final concentration of 5 μM completely ablated cleavage of the peptide substrate (not shown). Moreover, other recombinant proteins (non-proteolytic) expressed and purified in an identical fashion in our laboratory did not cleave Z-Phe-Arg-AMC (not shown).

Recombinant *Ac*-CP-2 is immunogenic in dogs

Dogs immunized with recombinant *Ac*-CP-2 formulated with different adjuvants produced IgG1 and IgG2 antibody responses as measured by ELISA using the recombinant protein (Figure 52). IgE titers were low (<1,500) and are not discussed further. The maximum IgG1 titers (geometric mean = 50,452) were induced by formulating *Ac*-CP-2 with ASO3. The maximum IgG2 titers (geometric mean = 78,294) were induced by formulating *Ac*-CP-2 with ASO2. Dogs immunized with adjuvant alone did not generate detectable immune responses until larval challenge, suggesting that antibodies to *Ac*-CP-2 (or a similar protease) are induced during natural infection with the parasite. *Ac-cp-2* mRNA was not identified from more than 9,000 expressed sequence tags generated from serum-stimulated (induced to feed) *A. caninum* L3 implying that the mRNA and protein are only expressed in the adult-blood feedingstages.

The increase in anti-*Ac*-CP-2 antibody titers in control dogs after L3 challenge (but before worms would have matured to adulthood) is likely due to secretion of antigenically related cysteine proteases by L3; the closest homolog of *Ac*-CP-2 from *A. caninum* L3 cDNAs (EST pb58a11.y1) shared 64% identity at the amino acid level. ASO2 and ASO3 adjuvants induced the greatest antibody responses, especially of the IgG2 subclass. ISA 70 and alum induced much weaker responses although the general pattern and duration of responses were similar to those induced by the ASO adjuvants.

Vaccination with *Ac*-CP-2 decreases fecundity of female hookworms

Dogs rapidly develop age- and exposure-related immunity to *A. caninum* [10]. We therefore observed egg counts from vaccinated animals up to 3 weeks post-challenge. At 3 weeks after larval challenge, a significant decrease in egg counts was observed in dogs vaccinated with Ac-CP-2 formulated with either ASO2, ASO3 or alum compared with dogs that were vaccinated with alum alone ($P \leq 0.05$) (Figure 53). Statistically significant differences between mean adult male worm burdens of dogs vaccinated with *Ac*-CP-2 and adjuvant alone were not seen (Table X). The greatest number of female worms was recovered from dogs immunized with alum alone (mean = 131); the smallest number of female worms was recovered from dogs immunized with *Ac*-CP-2/ASO3 (mean = 104). While the decrease in worm burdens in the latter group was noteworthy, the differences were not statistically significant.

Table X. Mean adult worm numbers recovered from the small and large intestines of dogs immunized with *Ac*-CP-2 formulated with different adjuvants or adjuvant alone.

	Small intestine		Large intestine	
Group	Male	Female	Male	Female
Ac-CP-2/ASO3	107	111	8	9
Ac-CP-2/ASO2	109	104	7	11
Ac-CP-2/ISA70	113	116	7	8
Ac-CP-2/alum	125	120	4	6
Alum	105	131	6	9

Vaccination with *Ac*-CP-2/ASO2 resulted in a lower proportion of female worms

Comparison of the proportions of male to female worms revealed that worms recovered from dogs vaccinated with *Ac*-CP-2/alum ($P = 0.05$) and *Ac*-CP-2/ASO2 ($P = 0.074$) had more male worms than female worms when compared with worms recovered from dogs immunized with adjuvant alone (Figure 54).

Vaccination with *Ac*-CP-2 protease stunts the growth of hookworms

At necropsy, all worms recovered from the vaccinated dogs were fixed in formalin. The lengths of 100 undamaged worms from each group were measured, and the mean lengths compared statistically. The mean lengths of female worms recovered from dogs vaccinated with *Ac*-CP-2/ASO2 ($P = 0.003$) and *Ac*-CP-2/ASO3 ($P = 0.033$) were shorter than that of worms recovered from dogs immunized with adjuvant alone (Table XI). Statistically significant differences in male worm lengths were obtained when male worms from dogs that received *Ac*-CP-2/ASO3 were compared with worms recovered from dogs immunized with alum alone ($P = 0.035$).

Table XI. Adult hookworms recovered from dogs that were vaccinated with *Ac*-CP-2 were shorter than those recovered from dogs immunized with adjuvant alone. *P* values compare the difference between each group that received the vaccine and the adjuvant alone group. N = number of worms measured.

Group	N	mean length (cm)	SD	*P* value*
Ac-CP-2/ASO3				
Female	100	0.534	0.22	0.033
Male	100	0.384	0.11	0.035
Ac-CP-2/ASO2				
Female	100	0.507	0.22	0.003
Male	100	0.432	0.12	0.844

Ac-CP-2/ISA70				
Female	100	0.572	0.21	0.567
Male	100	0.465	0.14	0.999
Ac-CP-2/Alum				
Female	100	0.558	0.24	0.567
Male	100	0.471	0.14	1.000
Alum only				
Female	100	0.612	0.28	---
Male	100	0.430	0.13	---

SD, standard deviation from mean. * indicates P-value for Dunnett t-tests in which one group is treated as a control and the test groups are compared against it.

Anti-*Ac*-CP-2 antibodies are ingested by and bind to the intestine of feeding hookworms

The site of anatomical expression of *Ac*-CP-2 within adult hookworms had not been previously reported. We therefore used sera from dogs immunized with *Ac*-CP-2/ ASO3 to localize expression to the brush border membrane of the intestine of adult worms (not shown). *Ac*-CP-1 on the other hand was shown by Harrop et al. [6] and confirmed by us here (not shown) to be expressed in the cephalic and excretory glands of the parasite, accounting for its presence in excretory/secretory products of adult *A. caninum* [6].

To determine whether vaccination of dogs induced circulating antibodies that bound to the intestinal lumen during infection, parasites were removed from vaccinated dogs, fixed, sectioned and probed with secondary antibody (anti-dog IgG conjugated toCy3) only. Worms recovered from dogs immunized with *Ac*-CP-2 (not shown) but not from dogs immunized with adjuvant alone (not shown) contained antibodies that were ingested with the blood-meal of the worm, and subsequently bound specifically to the intestine and intestinal contents.

IgG from dogs vaccinated with *Ac*-CP-2 neutralizes proteolytic activity *in vitro*

Purified IgG from dogs that were immunized with *Ac*-CP-2 was effective at neutralizing the catalytic activity of *Ac*-CP-2. Incubation of 50 ng of pooled IgG from dogs immunized with *Ac*-CP-2/ASO3 resulted in a 73% reduction in the cleavage of Z-Phe-Arg-AMC by 1.0 μg of *Ac*-CP-2 (Table XII). Fifty nanograms of IgG from dogs immunized with adjuvant alone resulted in a 3% reduction in proteolytic activity, implying that vaccination with *Ac*-CP-2 resulted in the production of antibodies that neutralized the function of the enzyme *in vivo*.

Table XII. Effect of pooled IgGs from vaccinated *(Ac*-CP-2/ASO3) and control (adjuvant alone) dogs on the proteolytic activity of recombinant *Ac*-CP-2 against the substrate Z-Phe-Arg-AMC. Values are expressed as mean percent reductions in proteolytic activity from triplicate experiments.

Treatment	*Ac*-CP-2 only	*Ac*-CP-2+ αCP-2 IgG	*Ac*-CP-2+ norm IgG	*Ac*-CP-2+E64
% reduction in proteolytic activity	0±0	73±3	3±2	100±0

DISCUSSION for EXAMPLE 13.

Here we describe vaccination of dogs with a recombinant cysteine protease that resulted in partial protection as measured by reduced fecal egg counts, stunting of adult worms, decreased proportion of female to male worms and the generation of protease-neutralizing antibodies that bind to the hookworm intestine in vivo. In the 1930's, the late Asa Chandler hypothesized that antibodies directed against critical parasite enzymes mediated a successful anti-helminthic immune response by preventing worms from digesting host proteins [11]. This is the first report of protective efficacy with a recombinant protease from a parasitic nematode, and provides support for Chandler's anti-enzyme theory.

Although secreted by *P. pastoris*, complete processing of recombinant *Ac-CP-2*

to yield a mature enzyme did not occur; nonetheless, proteolytic activity was detected in the purified protein. *P. pastoris* transformed with a cDNA encoding *F. hepatica* cathepsin L secrete a partially activated protease that also exhibits catalytic activity, however unlike *Ac*-CP-2, this enzyme completely auto-activated after 2 hours at pH 5.5 [12]. *Ac*-CP-2 displayed a broad pH range with optimal activity at pH 5-7, supporting earlier work that described an optimal pH range of 5-9 for ES products and somatic extracts of adult *A. caninum* using Z-Phe-Arg-AMC [3].

Hematophagous helminths require blood as a source of nutrients to mature and reproduce. Female schistosomes ingest 13 times as many erythrocytes and ingest them about nine times faster than male worms [13]. Moreover, mRNAs encoding hemoglobin-degrading proteases of schistosomes are over-expressed in female worms [14]. While similar studies have yet to be performed for hookworms, female hookworms are bigger than males and lay up to 10,000 eggs per day, implying that they have a greater metabolism and therefore demand for erythrocytes. *Ac*-CP-2 is expressed in the gut, and preliminary data described elsewhere [15] have shown that the enzyme is involved in hemoglobinolysis in the hookworm intestine. It is therefore not surprising that interruption of the function of *Ac*-CP-2 via the action of neutralizing antibodies had a deleterious effect on the growth of female worms and subsequent egg production.

Vaccination of livestock and laboratory animals with cysteine proteases of other nematodes as well as trematodes has resulted in anti-fecundity/anti-embryonation effects. Immunization of sheep with the intestinal brush border complex, H-gal-GP, confers high levels of protection (both anti-parasite and anti-fecundity) against *H. contortus* and at least three different protease activities, including cathepsin B cysteine proteases, have been detected in this extract. Immunisation of sheep with a cysteine protease-enriched fraction of *H. contortus* membranes resulted in 47% protection against adult worms and 77% reduction in faecal egg output [15]. To date, the success obtained in vaccinated laboratory animals with cysteine proteases purified from parasite extracts has not been reproduced with the corresponding recombinant proteins expressed in *Escherichia coli* presumably because the recombinant molecules are incorrectly folded (and catalytically inactive) and thereby fail to induce responses capable of inactivating native proteases [16].

Cysteine proteases are also efficacious as anti-trematode vaccines.

Vaccination of cattle with cathepsin L cysteine proteases of *F. hepatica* results in decreased embryonation and hatch rates of eggs in addition to decreased worm burdens [12]. While these studies were performed with native proteins, trials with yeast-expressed recombinant proteases are in progress [12]. Vaccine trials using a DNA construct for *S. mansoni* Sm32, an asparaginyl endopeptidase that is cysteine protease-like in function but unrelated in sequence to cathepsins L and B, induced an anti-fecundity effect in a murine model of schistosomiasis when administered as a DNA construct [17].

We recently described partial protection of hamsters against another hookworm, *Ancylostoma ceylanicu*m, by immunization with a larval antigen, *Ay*-ASP-2, as a model of human hookworm disease [1]. The orthologous protein from *A. caninum, Ac*-ASP-2, is expressed by the L3 stage of the parasite when it is stimulated to feed *in vitro* [18]. Vaccination with ASP-2 resulted in a 32% reduction in the number of worms that reached adulthood [1], and we envisage that a human hookworm vaccine would ultimately consist of multiple antigens targeting both the L3 and the blood-feeding adult-stage. The data presented here suggest that cysteine proteases lining the intestinal lumen of hookworms are a valid target in the design of vaccines against hookworm disease. We have identified other proteases of different mechanistic classes that line the intestinal brush border of adult hookworms where they digest host hemoglobin [7, 8, 15], and some of these molecules might also prove efficacious as recombinant vaccines against hookworm infection.

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Example 14. Canine Vaccine Trial with Antigens *Ac*-ASP-2, *Ac*-MEP-1, *Ac*-APR-1, and *Ac*-API

A canine vaccine trial was carried out to examine the protective efficacy of four antigens formulated with the adjuvant, ASO3. These antigens are *Ac*-ASP-2, *Ac*-MEP-1, *Ac*-APR-1, and *Ac*-API. The trial confirmed our earlier findings that ASP-2 is a promising vaccine antigen (based on both human serology and hamster animal trials). This was evidenced by reduction in worm number, worm size, and fecal egg counts. The trial also provided preliminary data that APR-1 and MEP-1 also offer promise as protective antigens.

Experimental Design and Methods

Vaccine study design and antigen-adjuvant formulation

The vaccine trial was designed to test *Ac*-API, *Ac*-ASP-2, *Ac*-MEP-1, *Ac*-APR-1 formulated with Adjuvant System 03 (AS03) obtained from GlaxoSmithKline (GSK). The rationale for selecting AS03 as an adjuvants is discussed elsewhere (Stoute et al, 1997). The ten purpose bred beagles were randomized into five arms: immunized with the adjuvant-formulated recombinant proteins or adjuvant only (control). To make six doses of antigen formulated with AS03, 600 g of recombinant protein (0.3 ml of antigen at a concentration of 2 $mg.ml^{-1}$) was mixed with 1.2 ml of 20mM Tris-HCl, 0.5 M NaCl, pH 7.9 and 1.5 ml of AS03; the contents of the tube were vortex mixed for 30 seconds then shaken at low speed for 10 minutes. Dogs were immunized with 100 g of formulated antigen in a final volume of 0.5 ml. ASO3 only control was prepared as described above, with PBS included instead of antigen. Formulation of GSK adjuvants were conducted according to the protocol provided by GSK. All

injections were performed intramuscularly (IM). Test and control articles were prepared on the day of injection. All animals received 4 immunizations approximately 3 weeks apart.

Hookworm infections and parasite recovery

A. caninum larvae were cultured from eggs collected in the feces of infected dogs. All hookworms in the infective challenge were approximately equal age (17±7 days post hatching). The species identity of the infective larvae were validated using PCR. All dogs were infected by the footpad method with the same dose of 500 L3 of *A. caninum* (Zhan et al, 2001). Larval challenge occurred on one of three consecutive days (at age 120 +/- 9 days). Fourteen-sixteen days after the final immunization, dogs were anaesthetized using a combination of ketamine and xylazine (20mg.kg^{-1} and 10mg.kg^{-1} respectively), and 500 *A. caninum* L3 in a final volume of 50 l were applied to the footpad.

Canine immunizations and antibody measurements

Beagles were immunized with formulated *Ac*-ASP-2 as previously described (Loukas et al, 2004). The vaccines were administered IM three times beginning at age 62 +/- 4 days. Boosts were administered to the dogs at intervals of 21 days. Blood was drawn at least once every 21 days and serum was separated from cells by centrifugation. Each animal's specific antibody response was evaluated by indirect ELISA using serum taken prior to the infective challenge (Loukas et al, 2004). Recombinant *Ac*-ASP-2 was coated onto microtiter plates at a concentration of 5 g.ml^{-1}. Dog sera were titrated between 1:100 and 1:2 × 10^6 to determine endpoint titers. Anti-canine IgG1, IgG2 and IgE antibodies conjugated to horse-radish peroxidase (Bethyl Laboratories) were used at a dilution of 1:1,000.

L3 skin penetration assays

Live *A. caninum* L3 were incubated with sera (neat) from immunized dogs then L3 were placed on canine skin to observe whether serum antibodies interfered with the penetration of skin *in vitro* (Williamson et al, 2003). Briefly, fresh skin from the ear of a dog was shaved, and approx. 4 cm^2 section of skin was stretched and sandwiched between 2 × 20 mL syringe barrels that were clamped together with bulldog clips. The lower syringe was filled to the top with PBS so that the buffer was in contact with the underside of the skin. One milliliter of PBS was placed on the skin for 15 min to check integrity of the skin. L3 (300 L3/group) were then incubated in 0.05 ml of PBS, pH 7.2, or undiluted serum from different vaccinated or control dogs for 30 min at 37 C. Each group of L3 were then placed on the upper side of the skin

(added to the 1.0 ml of PBS already present) and allowed to migrate for 30 min at RT. L3 that remained on the surface of the skin were collected and counted, by removing the remaining liquid with a pipette and washing the skin with 2 volumes of PBS. Each assay was performed in triplicate.

Expression and purification of the recombinant proteins

Ac-ASP-2. The cloning of *Ac*-ASP-2 is reported elsewhere (Hawdon et al, 1999).

Other antigens. Details of the cloning and/or expression of *Ac*-MEP-1 and *Ac*-APR-1 are reported elsewhere (Harrop et al, 1996; Brinkworth et al, 2001; Jones and Hotez, 2002; Hotez et al, 2002). Briefly, both of these proteins are hemoglobin-degrading proteasese from the alimentary canal of adult hookworms.

Antibody responses following immunization

The individual and geometric means of the IgG1, IgG2, and IgE antibody titers are shown in Table XIII.

Table XIII . Pre-challenge antibody titer to recombinant proteins following immunization

Ac-ASP2	IgG1	IgG2	IgE
A1	13,500	40,500	100
A2	13,500	13,500	100
A3	13,500	13,500	100
A4	13,500	40,500	100
A5	4,500	13,500	100
GEOMEAN	10,837	20,950	100

Ac-API	IgG1	IgG2	IgE
B1	4,500	40,500	N/A
B2	4,500	40,500	N/A
B3	1,500	40,500	N/A
B4	4,500	40,500	N/A
B5	4,500	13,500	100
GEOMEAN	3,612	32,511	100

Ac-MEP	IgG1	IgG2	IgE
C1	100	1,500	N/A
C2	100	500	N/A
C3	100	1,500	N/A
C4	N/A	1,500	N/A
C5	100	1,500	N/A
GEOMEAN	100	1,204	N/A

Ac-APR1	IgG1	IgG2	IgE
D1	N/A	500	N/A
D2	100	4,500	N/A
D3	N/A	100	N/A
D4	N/A	500	N/A
D5	N/A	500	N/A
GEOMEAN	100	562	N/A

The antibody responses to both ASP-2 and API were robust. However, only a single dog developed a substantial antibody titer to APR-1 and the overall antibody response to Ac-MEP-1 was weak. Closer analysis reveals that canines immunized with recombinant *Ac*-ASP-2/AS03 produced strong IgG1 and IgG2 antibody titers to recombinant *Ac*-ASP-2. The IgE titers to *Ac*-ASP-2 in the test canines were one log lower than the IgG1 and IgG2 titers. Dogs immunized with AS03 adjuvant alone did not generate detectable antibody responses to *Ac*-ASP-2 prior to larval challenge. Sera from dogs vaccinated with recombinant ASP-2 immunoprecipitated native ASP-2 from biotinylated *A. caninum* extracts (L3E), while sera from animals immunized with adjuvant alone did not precipitate any L3E proteins.

Reductions in adult worm burden following vaccination

The overall worm burden data in vaccinated vs. control (AS03) dogs is presented in Tables XIV and XV. Briefly, there was good consistency in the number of worms from each group, with the exception of the two hemoglobinase groups. Of all of the groups, the greatest mean worm burden reduction was among the ASP-2-vaccinated dogs, while the greatest median worm burden was in the MEP-1 vaccinated dogs.

Table XIV. Summary results of the worm burdens in vaccinated and control dogs

	Intestine		Colon		
	Male	Female	Male	Female	Total
A1	107	115	1	3	226
A2	85	72	9	8	174
A3	54	28	1	6	89
A4*	62	87	5	5	160
A5	72	101	4	4	181
Average	76	81	4	5	166
B1	54	86	3	3	146
B2	83	83	0	1	167
B3	80	66	2	12	160
B4	105	91	9	24	229
B5	115	91	10	21	237
Average	87	83	5	12	188
C1	65	64	0	0	129
C2	47	58	1	2	108
C3	131	153	2	7	293
C4	124	130	5	9	268

C5	49	50	8	12	119
Average	87	91	3	6	183
D1	65	76	0	1	168
D2	47	41	11	18	119
D3	131	69	3	3	146
D4	124	87	5	8	202
D5*	49	122	0	0	250
Average	83	79	4	6	177
E1	76	59	7	23	165
E2	103	119	1	3	227
E3	87	113	7	8	215
E4	99	82	7	8	196
E5	114	100	1	2	217
Average	96	95	5	9	204

* 1 intestinal worm of unknown gender

** 1 colon worm of unknown gender

Table XV. Mean and Medians of the worm burdens in vaccinated dogs relative to control (ASO3) dogs

Ac-ASP-2	Valid	5

	Mean	166.0000
	Median	174.0000
	Minimum	89.00
	Maximum	226.00
Ac-API		5
	Mean	187.800
	Median	167.0000
	Minimum	146.00
	Maximum	237.00
Ac-MEP		5
	Mean	183.4000
	Median	129.0000
	Minimum	109.00
	Maximum	293.00
Ac-APR-1		5
	Mean	177.0000
	Median	168.0000
	Minimum	119.00
	Maximum	250.00
ASO3 (adjuvant)		5
	Mean	203.8000
	Median	215.0000

Minimum	165.00
Maximum	226.00

The number of adult hookworms recovered from each of the ASP-2 vaccinated dogs was lower than the mean of the control dogs, with the exception of dog A1. This accounted for the overall mean worm burden reduction. Dog A3 in the ASP-2 group exhibited the greatest worm burden reduction for the entire study (58%). The worm burden reduction is statistically significant if dog A1 is removed ($P = 0.03$).

Among the MEP-1 vaccinated dogs, three out of the five exhibited significant protection as evidenced by worm burden reductions that exceeded 36%. However in two of the vaccinated dogs, the number of hookworms recovered exceeded the mean of the control dogs. These findings accounted for the large reduction in median worm burden, but not the mean.

Among the APR-1 vaccinated dogs, only dog D2 exhibited a significant reduction in worm burden (42%). Of interest, this was the only dog that acquired significant anti-APR-1 antibody titers following vaccination.

There was no remarkable reduction in worm burden following API vaccination. These data are also pictorially represented in Figure 55.

Reduction in Quantitative Egg Counts (QECs)

As shown in Fig. 56, there was a significant reduction in fecal eggs (fecundity) for the ASP-2, API, and APR-1 group relative to the control group. Fecal eggs were lowest in the ASP-2 vaccinated dogs. These data indicate that ASP-2 is an immunogenic molecule and a promising protective antigen. In addition, both MEP-1 and APR-1, each a adult hookworm hemoglobinase, show some promise at protection. MEP-1 vaccinations resulted in reduced median hookworm burdens, while in a single dog that developed anti-APR-1 antibody titers there was also a reduction in the number of adult hookworms. However, the overall low antibody titers in response to these molecules suggests that the results of this trial could be improved if the immunogenicity of each hemoglobinase was increased. Studies are underway to re-express the proteases in yeast in an effort to improve immunogenicity. Previously a third

hemoglobinase, CP-2, was successfully expressed in yeast, and shown to be immunogenic and protective (Loukas et al, 2004).

References Cited for Example 14.

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Example 15. Cloning, transformation and expression in *Pichia pastoris* of Na-asp-2

The purpose of this study was to identify the major orthologue of *Ac-asp-2* (Hawdon et al, 1999) from *Necator americanus*. To identify the orthologue, a cDNA library was prepared

as described in a research report published in the *Chinese Journal of Parasitology and Parasitic Diseases* (Zhan et al, 2000). Briefly, these L3 were obtained from hamsters infected with *Necator americanus* as described (Xue et al, 2003). The L3 have now gone through approximately 100 passages through hamsters, but were originally derived by coproculture from an *N. americanus* infected individual from Hunan Province (Xue et al, 2003). From 500,000 plaques screened using *Ac-asp-2* cDNA, only 2 positive clones were obtained. These two positive clones were subjected to DNA sequencing. Neither of these clones contained the full-length signal peptide.

Based on the sequence obtained, forward and reverse primers were selected (both with and without histag, and all with EAEAEF vector sequence) and synthesized (Integrated DNA Technologies, Inc., Coralville, IA). These primers were used to amplify *Na-asp-2* cDNA from the 1st strand Na-L3 cDNA. Na-L3 cDNA from mRNA extracted from L3 as described previously (Zhan et al, 2000). The L3 were obtained from golden hamsters infected with N. americanus as described previously (Xue et al, 2003). The PCR products were ligated into pPICZ A using EcoR1 and Xba1 sites. The ligation product was transformed into *E. coli* DH5 competent-rendered cells and the recombinants were selected by growing on LB-Zeocin plates. Eight colonies were picked from each transformation (with and without histag) and analyzed by PCR with vector primers. Each of the positive clones contained an insert of the predicted size. From two clones (one with histag and the other without) the plasmid was extracted and sent for DNA sequencing (Nevada Genomics Center).

The clones obtained did not contain the 5' end of the *Na-asp-2* cDNA, which encoded the N-terminus of the full signal peptide. Therefore, concurrent with the amplification of *Na-asp-2* cDNA from L3 it was of additional interest to unambiguously determine the 5' end of the full length clone. 5' RACE was conducted to obtained a full-length cDNA. This was done using the Gene Racer Kit from Invitrogen. Reverse primers for 5'RACE were selected from a portion of the cDNA encoding the C-terminus of Na-ASP-2. The primers were synthesized by IDT (Integrated DNA Technologies, inc., Coralville, IA). The 5' RACE clones were sequenced.

Rescreening: The purpose of rescreening was to make certain that there were no other major orthologues cDNAs to *Ac-asp-2* (SEQ ID NO: 19). We wished to make certain that our clone represented the only Necator ASP-2 found in L3. To conduct this work, two fragments of *Ac-*

asp-2 (SEQ ID NO: 19) cDNA were selected as probes based on the most conserved areas compared with *asp-2* from other species of hookworm. On this basis, 4 separate primers (two forward and two reverse) were synthesized. Two PCR products were amplified from *Ac-asp-2*/pPICZ A plasmid, labeled with ^{32}P-CTP, and hybridized under stringent conditions (65°C). Approximately 500,000 plaques were screened. Of the remaining colonies not worked up under the above section, the remaining colonies were pooled and re-screened with *Ac-asp-2* cDNA fragment.

Preparation of *Necator americanus* cDNA library and original screening of the library with *Ac-asp-2* clone.

The 2 positive clones obtained from heterologous library screening were subjected to DNA sequencing. The 2 positive clones were identical, each encoding an ORF with homology to *Ac-asp-2* (SEQ ID NO: 19)(designated as *Na-asp-2*). The *Na-asp-2* cDNA consisted of 731 bp with a 3' poly (A) tail. However, no 5- initiation codon was identified. The *Na-asp-2* cDNA encodies a predicted ORF of 206 amino acids, lacking initial Met at the N-terminus.

Amplification of *Na-asp-2* cDNA and ligation into pPICZ A

Copy DNA was amplified and successfully ligated into pPICZ A. Except for the histag at the C- terminus both of the two clones described above were identical. However, compared with the original sequence, there was a single mutation at position 119 from T to A. This resulted in a conservative substitution of a Leu to Met at amino acid 36 (the position is based on the original full length sequence).

5'-RACE to obtain the 5'-end of Na-asp-2 cDNA

Two clones were obtained by 5'RACE. Each contained the full length 5' end. These were designated 4a1 and 4a2. Sequence alignment of the full length clone revealed that 4a1 exhibited three bp changes. However, 4a2 showed no bp changes from the original cDNA clone. The predicted ORF of 4a2 revealed that *Na*-ASP-2 (SEQ ID NO: 69) exhibits approximately 60-70% amino acid identity with *Ac*-ASP-2 (SEQ ID NO: 12).

Re-screening of Na L3 cDNA library with *Ac-asp-2* cDNA fragment

16 positive clones were identified, each exhibiting different intensities. By secondary screening 6 single colonies were obtained, PCR amplified and sequenced. Four of the 6 clones were identical. One of the other clones was identical except at position 119 which exhibited a T to A mutation. A sixth clone was an entirely new gene product that represented a double-

domain pathogenesis related protein superfamily gene product (tentatively designated as *Na-asp-7*).

Repeat re-screening of Na L3 library

11 additional plaques were obtained, and their cDNA inserts were sequenced. At NGC. From this re-screening a total of 11 cDNAs were obtained. Each of these contained clearly defined Na-asp-2 sequences, which were identical except for variation at positions 55, 61, 66, 119, 193, 451, 496, and 650. However only two of these mutations resulted in amino acid alterations. These included, mutation at position 119, which resulted in either the appearance of a methionine or leucine and a mutation at position 66, which resulted in either the appearance of an alanine or glycine.

Transformation of *Na-asp-2* cDNA into *Pichia pastoris*

pPICZ A DNA containing the *Na-asp-2* coding sequence was prepared as described in RR-3001 and transformed into *Pichia pastoris* by electroporation. Four X33 strain colonies containing the presumptive *Na-asp-2* sequence were selected, and the presence of an insert was confirmed by PCR in three of the four colonies. To generate Research Seed Stock #1, colony number 2 was selected and grown in YPD, prior to storage at –70 C in YPD containing 25% glycerol. Subsequently, Research Seed Stock #1 was expanded twice, first in BMG, and subsequently in YPD. The DNA sequence and copy number was confirmed..

Transformation of *Na-asp-2* DNA into *Pichia pastoris*

pPICZ A DNA containing the *Na-asp-2* coding sequence (without histag and without N-terminal signal peptide) was prepared. Plasmid DNA containing the *Na-asp-2* coding sequence was transformed into *Pichia pastoris* as described in the Invitrogen Pichia expression manual (EasySelect™Pichia Expression Kit, Version F 000526; 25-0172).Zeocin (all transformants integrate at 5' AOX1 locus by single crossover). Briefly, the plasmid DNA was linearized with SacI and transformed into *Pichia pastoris* strain X33 and GS115 (Mut+) using electroporation. The transformants were plated on medium containing Zeocin (all transformants integrate at 5' AOX1 locus by single crossover; Mut phenotype is determined by the strain used).

Four colonies containing the presumptive *Na-asp-2* DNA were selected. Each of these was from the X33 strain. The presence of *Na-asp-2* DNA was confirmed by PCR using the following vector primers:

3'AOX1 5'-GCAAATGGCATTCTGACATCC-3' (SEQ ID NO: 74)

α-factor 5'-TACTATTGCCAGCATTGCTGC-3'(SEQ ID NO: 75)

The presence of an insert was confirmed by PCR in three of the four colonies.

Sequencing

Expanded research seed stocks were subjected to PCR using the vector primers described above, and subjected to DNA sequencing. DNA sequencing was conducted at the Nevada Genomics Center. *Na-asp-2* cDNA without signal peptides at the 5' end and with stop codon at the 3' end was cloned with the correct reading frame. There were no nucleotide mutations observed following *Na-Asp-2*/pPICZA transformation into *Pichia pastoris* X-33 and subsequent expansions. Only a single copy of *Na-asp-2* DNA was observed in both the research seed stock as well as colonies from the original Zeocin plate.

Determination of copy number

Genomic DNA was extracted both from colonies of the original Zeocin plate and expanded. This was done using the YeaStar Genomic DNA Kit (Zymo Research, Cat. # D2002). *Na-asp-2* probe was amplified from *Na-asp-2*-pPICZ αA plasmid and labeled with digoxin as described in PCR DIG Probe Synthesis Kit (Roche, Cat #1636090) and used to probe a Southern blot containing research seed clone DNA. Only a single copy of *Na-asp-2* DNA was observed in both the research seed stock as well as colonies from the original Zeocin plate.

References for Example 15

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Example 16. Cloning and Canine Vaccine Trial of Ac-GST.

Cloning

Cloning of the protein GST from *Ancylostoma caninum* was carried out by identifying homologous EST fragments of Ac-GST from *A. caninum* an L3 cDNA library by searching with WU-Blast2 using the Sj28 *(S. japanicum)* GST sequence. Primers were designed based on sequence information and the 5' and 3' ends of Ac-GST were isolated from *A. caninum* L3 cDNA by using GeneRacer kit (Invitrogen). A full length AcGST was obtained (Figure 57 A, SEQ ID NO: 76). The deduced amino acid sequence is shown in Figure 57B (SEQ ID NO: 77), and the alighment of the cDNA and the amino acid sequence is shown in Figure 57C. The coding sequence was clained into pPICZaA in the correct reading frame and the entire sequence was confirmed by re-sequencing from both strands.

Vaccine trial

A canine vaccine trial was completed with the following vaccine antigens tested as shown in Table XVI.

Table XVI. Canine vaccine trial description

Antigen	Expression Vector	Amount	Immunization	Aduvant	Route
Ac-CYS	*Pichia pastoris*	100 ug	Four	AS03	i.m.
Ac-MTP-2	*Pichia pastoris*	100 ug	Four	AS03	i.m.
Ac-GST	*Pichia pastoris*	100 ug	Four	AS03	i.m.
Adjuvant alone	-	-	Four	AS03	i.m.
Irradiated L3	-	1000 L3	Four	-	sc

Experimental Design and Methods

Purpose of the Study: The purpose of the study is to test the protective effects in laboratory dogs of vaccines containing various recombinant protein antigens derived from the canine hookworm *Ancylostoma caninum*. These include recombinant glutathione S transferase (GST),

cystatin, and MTP-2. All antigens are given in combination with the GSK adjuvant AS03 and are compared with an AS03 negative control. In addition, a fifth arm of the study employs radiation-attenuated (irradiated) infective larvae, a positive control.

Brief Outline of Study Sections: Purpose bred beagles were randomized into five groups. Four groups were given one of three candidate vaccines: Ac-Cystatin, Ac-MTP-2 and Ac-GST in combination with the adjuvant ASO3. One group will serve as the negative control receiving the adjuvant only. Another group will be a positive control immunized with irradiated infective larvae. Each animal's specific antibody response was evaluated by direct ELISA using serum taken prior to the infective challenge. Cellular immune responses were assessed by peripheral (blood cells) lymphoproliferative responses to specific recombinant antigens and/or crude extract of infective larvae (L3) or adult worms. Local cellular immune responses were performed post mortem with lymphocytes extracted from mesenteric lymph nodes and, if considered, spleen. After immunization, animals were challenged with a known number dose of infective third stage larvae of *A. caninum*. Quantitative ova counts, used to evaluate worm burden, were determined from fecal samples collected three times per week. These data were augmented by periodic blood values to monitor any anemia induced by the parasites and finally necropsy examination to confirm parasite load by counting, weighing, sexing and measuring adult worms. Tissues from different organs were examined macro and microscopically to assess any consequence of the vaccine, parasite or immune related lesions.

Test and Control Identification: Test and control articles were prepared for injection by mixing with the adjuvant. The experimental vaccines were comprised of the antigens Ac-Cystatin and Ac-MTP-2 and Ac-GST (expressed all in *Pichia pastoris*) in combination with the adjuvant ASO3.

Animals: The test and control animals were purpose bred, parasite naïve male beagles 56 ± 7 days of age. The trial was terminated twenty seven (27) days after parasite infection.

Administration of Test and Control Articles: The vaccines and adjuvant were administered intramuscularly (IM) three (3) times beginning when the dogs are 62 +/- 4 days old. The vaccines are boosted at 21+/- 3-day intervals. Four doses of the vaccines were given 21 days apart (days 0, 21, 42, and 52). The dogs were challenged percutaneously with 500 *A. caninum* L3, 14 days after the final vaccination.

Serum Samples for Quantitative ELISA Antibody Titers: Animals treated with vaccines containing foreign proteins develop an immune response resulting in an increased level of high affinity serum antibodies that are directed against the antigen. Quantitative ELISA using the antigenic proteins demonstrated the relative avidity of the immune response and provide a data set that can be applied to the identification and analysis of hookworm resistant animals. White blood cells were collected for immunological measurements nine (9) days after the last boost and at the time of euthanasia to address the cellular immune response status and cytokine production upon *in vitro* restimulation of lymphocytes.

Cellular Immunology Studies: Blood samples were taken from each animal at scheduled intervals by the veterinary technologist in heparanized tubes. Lymphoproliferation assays were performed *in vivo* on blood, and post mortem on blood and mesenteric lymph nodes.

Challenge Infection: *Ancylostoma caninum* larvae were cultured from the eggs collected in the feces of infected dogs. All hookworms in the infective challenge were approximately equal age (17±7 days). The species identity of the infective larva dose were validated using PCR DNA amplification and specific oligonucleotide primers. Overnight-collected feces of *A. caninum*-infected dogs were cultured, extracted and counted. All dogs were infected by the footpad method with the same dose (500 +/- 5%, of 3rd stage larvae of *A. caninum*. Larval challenge occurs on one of three consecutive days (at age 120 +/- 9 days) in 5 series. To minimize the difference in the infective L3 doses, each series included one dog from each (A-E group).

Larval Irradiation: The irradiated larvae vaccinations were performed in 2 subcutaneous doses of 1,000 L3 at each vaccination with intervals of 3 weeks between the doses. The challenge was performed 4 weeks after the second dose of vaccination. The irradiated larvae were obtained by irradiation with 40 krad from Cesium (137) as described in SOP 38.1 and a single batch of irradiated larvae was used for both doses of vaccinations.

Observations, Hematology, Serum Chemistries: The dogs were observed daily and were weighed at least every 18 days. Dogs that develop signs of moderate to severe anemia, diarrhea or develop a loss of body weight greater than 15% were observed more frequently. Anemia is considered mild (HCT 27-33%), moderate (HCT 21-26%) or severe (HCT<20). Prior to larval infection and at least one time every 21 days, blood samples are collected from all dogs. Blood withdrawal should be approximately equal in amount from all dogs. At this time, the mucous membranes are examined for pallor. A pre-vaccination blood sample was utilized for CBC

(hematology), serum chemistries, and a sample of serum will be frozen. The CBC includes: HCT (hematocrit), Hb (hemoglobin), MCHC (mean corpuscular hemoglobin content) and count of WBC (white blood cells), neutrophils, eosinophils, platelets, and monocytes/lymphocytes. Serum chemistries include: ALB (albumin), ALKP (alkaline phosphatase), ALT (alanine aminotranspherase), TBIL (total bilirubin), TP (total protein), Phos (phosphorous), Ca (calcium), BUN (urea nitrogen), CREA (creatinine), AMYL (amylase), Chol (cholesterol), & Glu (glucose). The first CBC was performed approximately five (5) ± two (2) after parasite infection.

Quantitative Egg Counts (QEC): Twelve days (12 ± 3) following parasite dosing, fecal examination for ova began and continued three times a week (generally M, W, F) until termination. The ova count method was performed according to the current version of SOP 7, which is a modification of the McMaster technique (Veterinary Clinical Pathology, 6th ed., 1994, page 9-10). The test was performed in the same way each time in order to quantitate the ova count. The ova were counted in a McMaster chamber under a binocular microscope and recorded. At this time fecal specimens were examined for the presence of gross blood and notation made on the animal observation form if blood is observed.

Adult Worm Count: Adult worms retained in the small and large intestines were collected. The small and large bowel will be collected and the small intestine will be suspended (the large bowel will not be suspended) in a container and incubated for at least two hours at 35 °C saline to collect the adult parasites. The adult worms were separated from the intestinal contents, counted, and preserved in formalin for subsequent count and analysis of sex, length and weight.

Results and Analysis

The different groups in this vaccine trial are labeled as follows: A or 1: Cystatin + AS03; B or 2: MTP-2 + AS03; C or 3: GST + AS03; D or 4: AS03; E or 5: Irradiated 3. As shown in Table XVII high antibody titers were achieved with each group following four immunizations.

Table XVII. Antibody titers in HV-12

Antigen/Dog	IgG1	IgG2
Cystatin		

A1	40,500	121,500
A2	121,500	364,500
A3	364,500	1,093,500
A4	121,500	364,500
A5	121,500	364,500
GEOMEAN	121,500	364,500
Ac-MTP2		
B1	121,500	364,500
B2	40,500	121,500
B3	121,500	121,500
B4	40,500	121,500
B5	40,500	40,500
GEOMEAN	62,850	121,500
GST		
C1	13,500	40,500
C2	13,500	121,500
C3	13,500	40,500
C4	13,500	40,500
C5	13,500	40,500
GEOMEAN	13,500	50,452
L3 Extract		
E1	13,500	4,500

E2	13,500	1,500
E3	13,500	4,500
E4	13,500	500
E5	13,500	4,500
GEOMEAN	13,500	2,328

The adult hookworms recovered from each of the vaccinated dogs is shown in Table XVIII.

Table XVIII. Adult Hookworm Worm Counts in HV-12

	Intestine			**Colon**			
	Male	Female	Unk. Sex	Male	Female	Unk. Sex	Total
A1	81	85		1	0		167
A2	38	29		0	0		67
A3	48	67	1	3	5		124
A4	36	41		0	2		79
A5	44	56		0	0		100
Average	**49**	**56**					**107**
B1	64	71		11	10		156
B2	49	51		2	3		105
B3	41	41		1	2		85
B4	64	65		9	15		153
B5	87	73		1	0		161
Average	**61**	**60**					**132**
C1	50	81		0	0		131
C2	34	26		4	3		67

C3	19	36	0	0	55
C4	33	36	7	4	80
C5	33	41	2	5	81
Average	**34**	**44**			**83**
D1	49		0	1	113
D2	27	29	0	0	56
D3	62	61	0	2	125
D4	75	82	0	2	159
D5	108	119	3	2	232
Average	**64**	**71**			**137**
E1	7	10	4	9	30
E2	43	40	0	1	84
E3	28	118	4	5	155
E4	20	24	0	0	44
E5	12	15	3	5	35
Average	**22**	**41**	**2**	**4**	**70**

Although a promising trend was noted in the GST and cystatin vaccinated group (40 and 51 percent reduction relative to AS03 controls, respectively), for this trial the variance was too great for the small sample size and that only the IrL3 is statistically significant and, then only when the Dunnett 2-sided post hoc test (the standard for clinical trials) was used. However as shown in the Appendix, if an outlier is removed from the control group, statistical significance is obtained. An outlier is defined as an observation far from the rest of the data; it may represent valid data or a mistake in experimentation, data collection, or data entry. An outlier can have an extremely large effect on when testing for differences of means. While it is common to remove outliers, it must be done with some rules and with consistency. There is

statistical significance for both GST and IrL3, by using the 5% Trimmed Mean. This is the arithmetic mean calculated when the largest 5% and the smallest 5% of the cases have been eliminated. Eliminating extreme cases from the computation of the mean results in a better estimate of central tendency, especially when the data are non-ormal. This is common, well-accepted, and a method preferential to removing outliers because it is done by the statistical program itself. The results from an SPSS output for GST, IrL3, and Control groups are shown below in yellow for the 5% trimmed mean The results for the t-tests were: GST vs. Control (t = 1.6874; p = 0.0458); IrL3 vs. Control (t= 1.8851; p = 0.0297). A comparison of the resulting hookworm counts is given in Figure 59.

As shown in Figure 60, there was also a reduction in the mean and median hookworm quantitative egg counts in dogs receiving L3 irradiated and Ac-GST.

This example shows that high antibody titers were produced to each of the recombinant antigens. After larval challenge, both GST and irradiated L3 vaccinated groups exhibited high levels of worm burden reduction (41 and 50%, respectively). However, because of high variation within the control group, the worm burden reduction was statistically significant with either removal of outliers or using trimmed means. In addition there was significant reduction in quantitative egg counts. These studies confirmed the protection afforded by irradiated L3 and indicate that GST is a promising vaccine antigen.

Example 17. Hamster vaccine trial

These studies were undertaken to confirm the protective effects of Ay-ASP-2 observed in HamV-3 (Goud et al, 2004). The results confirm that ASP-2 is a protective antigen, both in terms of worm burden reduction and in worm fecundity. In addition there was less blood loss among the ASP-2 vaccinated group. The study also found that ASP-1 had greater protective efficacy than observed in HamV-3. The results also found that the addition of MTP to the vaccine cocktail increases the protective effect.

Experimental Design and Methods

Purpose of the Study: The purpose of the study is to test the protective effects in laboratory hamsters of vaccines containing various recombinant protein antigens derived from hookworms and other parasites, against hookworm infection.

Brief Outline of Study Sections: Purpose bred Syrian hamsters are randomized into eight groups. Seven groups will receive candidate vaccines: ASP-1, ASP-2, MTP, and Irradiated

larvae. One group receives only the adjuvant (Quil A) as experimental control. Each animal's specific antibody response is evaluated by direct ELISA using serum taken prior to the infective challenge. After the immunized animals demonstrate a positive immune response to the vaccines, they are challenged with a known number dose of infective third stage larvae of *A. ceylanicum.* Quantitative ova counts, used to evaluate worm burden, are made from fecal samples collected twice after larval challenge. Also, hemoglobin levels will be tested to detect anemia caused by the blood loss during adult hookworm infection. The final report will evaluate the data and provide conclusions regarding each vaccine's effectiveness both in terms of worm burden and blood loss.

Positive Result Indicators: A successful positive result in this study will be a demonstrated increase in specific antibody titers in immunized animals and protection against hookworm burden and hookworm-associated blood loss. The hemoglobin test detects anemia caused by the blood loss during adult hookworm infection. The experimental control data obtained from un-immunized animals will serve as a basis for evaluating the success of the study and also to check any parasitic infection.

Test and Control Identification: The antigens are ASP-1, ASP-2, and MTP. The antigen-adjuvant combinations will be ASP-1+Quil A, ASP-2+Quil A, MTP+Quil A, ASP-1+ASP-2 +Quil A, ASP-2+MTP+Quil A and ASP-1+MTP+Quil A. One group, which receives irradiated L3 serve as the positive control. The negative control group will receive only Quil A. Details about the antigen and the adjuvants will be included in the study records when they become available.

The test articles are diluted to provide a dose of 0.025mg of antigen in 200ul of antigen-adjuvant mixture per animal per injection. All injections will be performed intramuscularly (i.m). The prescribed volume dose information is recorded by Dr. Ghosh. Fresh preparation of antigens will be made the day of injection.

Selection and Justification of Test System: Hamsters are selected as the test system because they are susceptible to infection by a hookworm species that causes a serious but often non-fatal disease. Hamsters make an excellent model because hookworm- induced anemia caused by *A. ceylanicum* is better reflected in hamsters. Previous studies have documented there are many parameters associated with hookworm induced anemia that contribute to the quantitative evaluation of the vaccine study success.

Animals: The test and control animals will be purpose bred, parasite naïve, 23 ± 2 days old and 50 ± 5 gm body weights on arrival. Following 5-9 days quarantine, the hamsters are started on the vaccination schedule. The hamsters will be identified by a small metallic ear tag plate, each of which contain a number for the identification of the hamster. Hamsters are randomized into five (6) vaccine test groups containing ten (10) hamsters each and two (2) control group of ten (10) hamsters. The hamsters are then assigned permanent hamster-study numbers (e.g. HamV-IV) as follows: HamV-IV (*A. ceylanicum* Vaccine Trial IV), vaccine or control groups A, B, C, D E, F, G and H. Each hamster will have unique Ear Tag number viz., 301. Attempts will be made to treat each hamster in the same manner. Each hamster on a trial will receive the same treatment, housing, dose of larvae and diet.

Administration of Test and Control Articles: The vaccines and adjuvant are administered intramuscularly (IM) three (3) times beginning when the hamsters are 28 ± 2 days old. The vaccines are boosted at 21 days (3 weeks) intervals.

Serum Samples for Quantitative ELISA Antibody Titers: Animals treated with vaccines containing foreign proteins are expected to develop an immune response resulting in an increased level of high affinity serum antibodies that are directed against the antigen. Since the hookworms feed on blood, antibodies in the host circulatory system are likely to come in contact with the parasite. If these antibodies recognize an antigen that is essential for initiation or maintenance of the parasitic state, immune reactions may exert a protective effect that causes a significant change in the critical infection parameters (i.e. egg counts, blood values, worm number, or worm size). Quantitative ELISA using the antigenic proteins will demonstrate the relative avidity of the immune response and will provide a data set that can be applied to the identification and analysis of hookworm resistant animals.

Challenge Infection: *Ancylostoma ceylanicum* larvae are cultured from the eggs collected in the feces of infected hamsters by qualified technicians in the Dr. Hotez lab. All hookworms in the infective challenge are approximately equal age (10±5 days). The species identity of the infective larva dose is validated, using PCR DNA amplification and specific oligonucleotide primers. All hamsters are infected by orally with the same dose of 100 +/- 10 3rd stage larvae of *A. celyanicumfs*. Larval challenge occurs on the same day for all hamsters (at age 82 ± 2 days).

Clinical Observations: The hamsters are observed daily and are weighed at least every 7 days post-challenge. Hamsters that develop signs of moderate to severe anemia, or develop a loss of

body weight greater than 15% are observed more frequently. Prior to larvae infection and at least one time every 7-10 days, blood samples are collected from all hamsters. Blood withdrawal should be approximately equal in amount from all hamsters and at this time, the stool will be checked for blood.

A pre-vaccination blood sample will be examined for Hemoglobin; a sample of serum will be frozen. A hemoglobin test is performed 6-10 days and 12-18 days post-challenge. Samples of serum will be collected from each hamster; pre-immune, after 2 boosts, and after larval challenge. These samples will be labeled with unique identifiers (nature of the specimen, study-hamster number and collection date) and frozen for possible future analysis.

Quantitative Egg Counts (QEC): One week following parasite dosing, fecal examination for ova begins and will be repeated once a week until the study is terminated. The ova count method will be performed according to the SOP, which is a modification of the McMaster technique (Veterinary Clinical Pathology, 6th ed., 1994, page 9-10). The test will be performed the same way each time in order to quantitate the ova count. Fecal specimens from the hamsters will be identified by the hamster study number and the unique hamster identification number. The ova are counted in a McMaster chamber under a binocular microscope and recorded.

Termination: Hamsters that appear to be suffering (and the pain cannot be relieved) or become moribund are euthanized. All hamsters that are euthanized or die spontaneously are necropsied. The study is terminated 4 to 5 weeks (+/– 3 days) after parasite infection. Three groups of hamsters will be euthanized on each day of necropsy with one of the groups being a group of control hamsters. Halothane will be used for euthanasia.

Necropsy: A complete necropsy is performed. Lesions are described, the entire small and large intestine is collected, and tissues are fixed in formalin. The ear tags will be retained with the tissues in formalin.

Adult Worm Count: The small and large intestines are collected and incubated in a petri dish for a few minutes at 37°C± 7° in saline to facilitate the collection of adult parasites. The adult worms are separated from the intestinal contents, counted, and preserved in formalin for subsequent count and analysis of sex.

Statistical Methods: Both parametric and non-parametric tests will be used to analyze the data. Statistical comparisons for each outcome variable will be performed at the two-sided =

.05 level of significance. Variables that will be analyzed are: Number of worms in the intestine and the colon; Egg counts per gram; Antibody titers; Hemoglobin and Body Weight of hamsters.

Results and Discussion.

The Geometric mean of antibody titers for each group under study are given in Figure 77A, and the reduction in worm burden is depicted in Table XIX and graphically in Figure 77B. As can be seen, the results confirm that ASP-2 is a protective antigen, both in terms of worm burden reduction and in worm fecundity. In addition, there was less blood loss among the ASP-2 vaccinated group. The study also found that ASP-1 had greater protective efficacy than observed in HamV-3. The results also found that the addition of MTP to the vaccine cocktail increases the protective effect.

Table XIX. Hookworm burden reductions following vaccination with recombinant antigens or irradiated *A. ceylanicum* L3 followed by *A. ceylanicum* L3 challenge.

Experimental Groups	Adult Hookworms Mean (Median) ± 1 SD	Percentage Reduction Relative to Quil A	*P* (one sided)
Ay-ASP-1	30.9 (36.0) ± 13.8	36.8	0.003*
Ay-ASP-2	33.2 (39.5) ± 15.2	32.1	0.005*
Ay-MTP	35.3 (40.0) ± 19.6	27.8	0.026
Ay-ASP-2 + Ay-ASP-1	43.7 (43.5) ± 20.4	10.6	0.29
Ay-ASP-2 + Ay-MTP	31.4 (31.5) ± 13.4	35.8	0.002*
Ay-ASP-1 + Ay-MTP	27.0 (29.0) ± 20.0	44.8	0.011
Quil A (Adjuvant control)	48.9 (53.0) ± 12.9	-	-
Irradiated L3	6.8 (4.5) ± 5.5	86.1	0.001*

* $P \leq 0.007$ is considered significant after Bonferroni correction

Table XX shows data regarding hookworm egg reduction.

Table XX. Hookworm eggs (EPG) reductions following vaccination with recombinant antigens or irradiated *A. ceylanicum* L# follwoed by *A. ceylanicum* L3 challenge.

Experimental Groups	EPG Mean ± SD	Percent reduction relative to Quil A
Ay-ASP-1	912.5 ± 17.7	59.0
Ay-ASP-2	1175.0 ± 176.7	47.2
Ay-MTP	1275.0 ± 777.8	42.7
Ay-ASP-2 + Ay-ASP-1	1312.5 ± 1007.6	41.0
Ay-ASP-2 + Ay-MTP	1012.5 ± 512.7	54.9
Ay-ASP-1 + Ay-MTP	1025.0 ± 707.11	53.9
Quil A (Adjuvant control)	225.0 ± 1343.5	-
Irradiated L3	25.0 ± 0.000	98.9

Additional date concerning blood loss is given in Table XXI.

Table XXI. Hemoglobin reduction at necropsy relative to hemoglobin at the time of experimental infection of hamsters with *A. ceylanicum* L3, and its comparison with control group.

Experimental Groups	Hb % change Mean (Median) ± 1 SD	Percentage Hb Increase Relative to control (QuilA)	P (one sided)
Ay-ASP-1	-13.3 (-20.0) ± 20.0	32.7	0.022

Ay-ASP-2	-9.2 (-13.3) ± 16.0	55.2	0.003*
Ay-MTP	-3.5 (-11.9) ± 30.0	59.9	0.018
Ay-ASP-2 + Ay-ASP-1	-17.9 (-23.2) ± 24.3	21.9	0.11
Ay-ASP-2 + Ay-MTP	-15.2 (-17.2) ± 10.3	42.1	0.008
Ay-ASP-1 + Ay-MTP	0.8 (5.4) ± 18.0	118.2	0.002*
Quil A (Adjuvant control)	-29.7 (-32.5) ± 14.8	-	-
Irradiated L3	13.7 (21.4) ± 17.6	172.1	<0.0001*

Minus sign means percentage decrease

* $P \leq 0.007$ is considered significant after Bonferroni correction

Figure 78B display this data graphically.

Table XXII gives spleen weights of hamsters per experimental group.

Table XXII. Spleen weights of hamsters per group.

Experimental groups	Spleen weights (gr) Mean (Median) ± 1 SD
Ay-ASP-1	0.48 (0.48) ± 0.10
Ay-ASP-2	0.44 (0.47) ± 0.13
Ay-MTP	0.42 (0.45) ± 0.13
Ay-ASP-2 + Ay-ASP-1	0.45 (0.48) ± 0.13
Ay-ASP-2 + Ay-MTP	0.42 (0.44) ± 0.08
Ay-ASP-1 + Ay-MTP	0.44 (0.42) ± 0.12
Quil A (Adjuvant control)	0.47 (0.45) ± 0.06

Irradiated L3	0.23 (0.23) ± 0.09

Table XXIII shows data concerning body weight reduction of hamsters at necropsy.

Table XXIII. Body weight reduction fo hamsters at necropsy relative to their weight at the time of experimental infection with *A. ceylanicum* L3, and its comparison with control group.

Group	Body Weight % Reduction Maen (Median) ± 1 SD	*P*
Ay-ASP-1	3.4 (4.4) ± 5.9	0.02
Ay-ASP-2	4.2 (4.9) ± 4.8	0.03
Ay-MTP	4.0 (5.4) ± 6.0	0.04
Ay-ASP-2 + Ay-ASP-1	4.7 (5.4) ± 3.9	0.04
Ay-ASP-2 + Ay-MTP	4.0 (5.2) ± 4.1	0.04
Ay-ASP-1 + Ay-MTP	5.5 (7.2) ± 4.4	0.20
Quil A (Adjuvant control)	7.9 (7.8) ± 3.0	-
Irradiated L3	4.2 (5.2) ± 3.3	0.02

In addition, Figure 80A and B illustrate IgG titers vs median worm burden (A) and EPG (B).

This example demonstrates that a significant reduction in worm burden was observed for animals vaccinated with ASP-1, ASP-2, ASP-2 + MTP, and irradiated L3. In these animals, an overall reduction in egg count from 41% to 98.9% was observed. Significantly higher hemoglobin was observed in animals vaccinated with ASP-2, ASP-1 + MTP and irradiated L3 ($P \leq 0.007$, and for ASP-2 + MTP, $P = 0.008$). Further, a statistically significant negative correlation was observed between spleen weight and hemoglobin ($P < 0.001$).

Reference Cited in Example 17.

Goud GN et al. 2004. Cloning, yeast expression, isolation, and vaccine testing of recombinant Ancylostoma-secreted protein (ASP)-1 and ASP-2 from Ancylostoma ceylanicum. Journal of Infectious Diseases 189: 919-29

While the invention has been described in terms of its preferred embodiments, those skilled in the art will recognize that the invention can be practiced with modification within the spirit and scope of the appended claims. Accordingly, the present invention should not be limited to the embodiments as described above, but should further include all modifications and equivalents thereof within the spirit and scope of the description provided herein.